Structural and functional characterization of the budding yeast Mus81-Mms4 complex

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirement for the Degree of Master of Science in the Department of Microbiology and Immunology University of Saskatchewan

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ABSTRACT

The *Saccharomyces cerevisiae* Mms4 and Mus81 proteins are required for repairing DNA alkylation damage, but not damage caused by ionizing radiations. Previous studies have demonstrated that Mms4 and Mus81 form a specific complex *in vivo*, which functions as an endonuclease specific for branched DNA molecules.

In an effort to further understand the role of the Mus81-Mms4 complex *in vivo*, the structural and functional characteristics of this complex were investigated in this study. The epistatic analysis revealed that *RAD52* was epistatic to *MMS4* with respect to killing by methyl methanesulfonate (MMS), suggesting that *MMS4* is involved in the *RAD52* dependent homologous recombinational repair pathway. However, the *mms4Δ rad51Δ*, *mms4Δ rad54Δ* and *mms4Δ rad50Δ* double mutants showed more sensitivity to MMS than either corresponding single gene disruptant. Since Rad51 and Rad54 are required to form the Holliday junction during recombinational repair pathway, it is unlikely that the Mus81-Mms4 complex functions as a Holliday junction resolvase *in vivo*.

The role of *MMS4* in DNA damage induced mutagenesis has been investigated. Deletion of *MMS4* had no obvious effects on damage-induced basepair mutations, but increased frame-shift mutations by 3 fold when the yeast cells were treated with MMS. This suggests that the Mus81-Mms4 complex plays a role in limiting the damage-induced frame-shift mutagenesis.

Through a yeast two-hybrid assay, Mus81 and Mms4 have been demonstrated to form a stable and specific complex *in vivo*. This result is consistent with previous studies. To localize the domains of the Mms4 and Mus81 proteins involved in herterodimer formation, a series of deletion mutants were constructed for the yeast two-hybrid assay.

The Mus81-binding domain of Mms4 was mapped to the extreme C-terminal region between amino acids 598-691. The Mms4-binding domain of Mus81 was mapped to a domain between amino acids 527-632. The results from co-immunoprecipitation experiment were consistent with those from the yeast two-hybrid assay. The Mms4-1 (Gly173Arg) protein was found to lose its interaction with Mus81, and this kind of amino acid substitution is very likely to alter the three-dimension structure of the protein. Thus we hypothesize that the three-dimensional structure is also important for Mms4 to interact with Mus81.

By studies on green fluorescent protein (GFP) fusion proteins and their subcellular localization, we demonstrated that Mms4 and Mus81 are nuclear proteins. When the putative nuclear localization sequence 1 (residues 244-263) in Mms4 was deleted, the truncated protein lost the ability to enter the nucleus. On the contrary, deletion of the putative nuclear localization sequence 2 (residues 539-555) had no effect on the localization of the protein. Furthermore, the nuclear localization of Mus81 was proven to be independent of its interaction with Mms4, and the N-terminal half of Mus81 is necessary and sufficient for its localization to the nucleus.

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To my family

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LIST OF ABBREVIATION

aa	amino acid
Amp	ampicilin
AP	apurinic/apyrimidinic
AT	ataxia telangiectasia
ATLD	ataxia-like disorder
ATM	ataxia telangiectasia
BER	base excision repair
β-gal	β-galactosidase
BIR	break-induced replication
CIP	calf intestinal alkaline phosphatase
CS	cockayne syndrome
DAPI	4’ 6,-diamidino-2-phenylindole
ddH_2O	double distilled water
DMS	dimethyl sulfonate
DMSO	dimethyl sulfoxide
DSB	double-stranded DNA break
dsDNA	double stranded DNA
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
EtBr	ethidium bromide
GFP	green fluorescent protein
GGR	globe genomic repair
HHR	homologous recombination pathway
Ile	isoleucine
IR	ionizing radiation
Kb	kilobase pair
kDa	kilodalton
Leu	leucine
Lys	lysine
MAT	mating type locus
Met	methionine
MMR	mismatch repair
MMS	methyl methanesulfonate
MNNG	*N*-methyl-*N’*-nitro-*N*-nitrosoguanidine
NBS	nijmegen breakage syndrome
NER	nucleotide excision repair
NHEJ	non-homologous end joining

NLS	nuclear loclalization sequence
OD	optical density
ORF	open reading frame
PBS	phosphate buffer saline
PNCA	Proliferating Cell Nuclear Antigen
RF	replication factor
RPA	replication protein A
S. cerevisiae	*Saccharomyces cerevisiae*
SD medium	synthetic dextrose medium
SDS	sodium dodecyl sulfate
SDSA	synthesis-dependent strand annealing
S. pombe	*Saccharomyces pombe*
SSA	single-strand annealing
SSB	single-stranded DNA break
ssDNA	single stranded DNA
TCR	transcription coupled repair
Trp	tryptophan
TTD	trichothiodystrophy
Ura	uracil
UV	ultraviolet radiation
Wt	wild type
XP	xeroderma pigmentosum

CHAPTER ONE

INTRODUCTION

1.1 DNA damage

DNA is the carrier of genetic information in all organisms. It was assumed that DNA should be extraordinarily stable in order to maintain a high degree of fidelity. Actually, the primary structure of DNA is quite dynamic and subject to constant change. Chemical modification can occur at many parts of the polynucleotide chain: the sugar-phosphate backbone, the glycosylic bond between the sugar and the nitrogen base, and certain atoms of the nitrogen or oxygen base. Most modifications of the molecular structure of DNA and the alterations in nucleotide sequence are appropriately considered to be damages to DNA. Many of these changes arise as a consequence of errors introduced during replication, recombination and DNA repair itself. Some changes arise from reactions between DNA and chemical compounds and physical agents. For convenience, damage can be classified into two major classes: endogenous and environmental.

1.1.1. Endogenous DNA damage

DNA damage can arise spontaneously from errors in basic cellular processes such as replication and from the byproducts of cellular metabolism. For example, estimates of the daily number of DNA lesions in a human cell range from 100- 500 spontaneous deaminations to 20,000-40,000 single strand breaks (Ames and Shigenaga, 1992).

DNA replication is the chief source of the DNA alteration. Most replication errors can be attributed to the limited proofreading ability of DNA polymerases and modified bases on the template caused by endogenous DNA damaging agents. In addition, errors are easily introduced due to dNTPs pool imbalance (Kunkel et al., 1982) or the absence of certain accessory protein such as ssDNA binding protein (Kunkel et al., 1979).

Cytosine, adenine and guanine contain exocyclic amino groups. The loss of these groups occurs spontaneously in pH- and temperature-dependent reaction of DNA. Deamination of cytosine results in the formation of uracil, which preferentially base-pairs with adenine, and causes a transition mutation. The deamination product of adenine is hypoxanthine, which can base-pair with cytosine in replication, and is potentially mutagenic. After deamination, guanine is changed to xanthine, which arrests DNA replication and is therefore lethal (Friedberg et al., 1995).

Generation of abasic (apurinic or apyrimidinic) sites is a very common type of spontaneous DNA base damage. Abasic sites occur when glycosylic bonds break, resulting in the loss of the nitrogen base. An abasic site is a strong replication-blocking lesion (Sagher and Strauss, 1983), and prone to cause strand break (Lindahl, 1993). Abasic sites can be mutagenic due to error-prone polymerase bypass of the abasic site with random base incorporation (Nelson et al., 1996).

Strand breaks also occur spontaneously at quite significant frequencies in the cell. During the cell cycle, spontaneous strand breaks may arise by topoisomerase-mediated DNA cleavage. For instance, Topo I generates reversible single-stranded DNA break (SSB) and Topo II generates reversible double-stranded DNA breaks (DSB) during

mitosis and meiosis. During replication, any SSBs in the parental strand can be easily converted into DSBs upon arrest of the replication fork at this lesion. Furthermore, defects in the maturation of Okazaki fragments may lead to the accumulation of DSBs (Lieber, 1997; Tishkoff et al., 1997).

1.1.2 DNA damage caused by environmental DNA damaging agents

1.1.2.1. DNA damage caused by physical agents

When DNA is exposed to UV light, adjacent pyrimidines become covalently linked by the formation of a four-member ring structure resulting from saturation of their respective C5, C6 double bonds. Another major product of UV irradiated DNA is a 6-4 photoproduct. This dimer is a noncyclobutane type of di-pymidine photoproduct, in which there is bond formation between the C6 and C4 of adjacent pyrimidine bases. In addition, UV light can cause different forms of base damage, DNA-protein crosslinks, and DNA strand breaks, although these lesions are much less prominent (Friedberg et al., 1995).

DNA damage from ionizing radiation (IR) can occur from the direct deposition of energy to DNA, as well as indirectly through the interaction of reactive species formed by the radiation. IR causes a wide spectrum of chemically different lesions in DNA. Ionizing radiation induces strand breaks directly, and most of the lethal effects can be attributed to these lesions. 100 Rads of IR can induce 600-1000 single SSBs and 16-40 DSBs in mammalian cells (Ward, 1988). SSBs are initiated by radical formation at deoxyribose following the loss of the hydrogen atoms. This can result from a direct ionization event or form abstraction by an $^{\cdot}OH$ radical. Subsequently the radical can

react with oxygen and form a preoxy radical. Details of the sequence of reactions leading to strand breaks are not entirely clear. Additionally, it is not known whether DSBs are caused by radical transfer to the opposite strand or whether they are a consequence of multiple radical attacks leading to independent SSB. If SSBs on the opposite strands are sufficiently close, a DSB will result (Friedberg et al., 1995). The majority of these strand breaks bear damaged termini, so a simple religation is impossible. IR can result in the formation of intermolecular DNA cross-links as minor products of DNA damage. IR also causes chemically modified base and sugar moieties. For example, hydroxyl radicals caused by IR attack the C5 and C6 double bond of thymine, leading to thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) formation (Friedberg et al., 1995).

1.1.2.2. DNA damage caused by chemical agents

DNA alkylating agents represent a large group of toxic chemicals present in the environment. Theses agents react with DNA by covalently binding to nucleophilic sites on the DNA molecule leading to DNA-alkyl adducts. Alkylating agents are classified into either S_N1 or S_N2 reacting agents based on their reaction. The S_N2 reagents undergo rapid transfer of the alkyl group on nucleophilic attack on DNA, whereas the S_N1 chemicals usually degrade to reactive intermediates that react with DNA. Methyl methanesulfonate (MMS), a prototype alkylating agent, reacts by an S_N2 mechanism. MMS alkylates DNA primarily at N^7-guanine and N^3-adenine, the latter resulting in lethal lesions (Lindahl et al., 1988). A number of methylating agents such as *N*-methyl-*N*-nitro-*N*'-nitrosoureas (MNNG) form O-alkyl lesions such as O^6-MeG and O^4-MeT, which can pair with deoxythymine and deoxyguanine, respectively, and form mismatches leading to transition mutations (Eadie et al., 1984; Loveless, 1969; Preston et al., 1986).

Interstrand DNA cross-links represent an important class of chemical damage to DNA, since they prevent DNA strand separation and hence can constitute complete blocks to DNA replication and transcription. A number of chemical agents such as mitomycine and nitrogen mustard can cause interstrand DNA cross-links (Friedberg et al., 1995).

1.2. DNA repair pathways

Some lesions mentioned above are of little biological significance because they do not interfere with cellular processes such as transcription and replication, whereas others must be repaired to avoid the consequences of mutation or cell death. The repair of damage to DNA is essential to the survival of the cell and the health of the organism. Indeed cells have evolved many DNA repair pathways to deal with a diverse range of DNA lesions and adducts. The major pathways include base excision repair, mismatch repair, nucleotide excision repair, postreplication repair and recombination repair. Defects in these pathways result in increased frequencies of cell death, mutations and chromosomal aberrations (Friedberg et al., 1995). From a medical standpoint, individuals with defects in these DNA repair pathways are often predisposed to cancers. Meanwhile, DNA damage repair is closely linked to aging. Empirical evidence from many lines of research suggest that ageing is a process of gradual accumulation of DNA damage in cells and tissues of the body, leading eventually to frailty and increased risk from a spectrum of age-associated diseases (Kirkwood, 2002).

1.2.1. Base excision repair pathway

Base excision repair (BER) is a process to remove damaged bases from the DNA and replace them with pristine sequence. Base excison repair normally repairs DNA damage caused by both endogenous and exogenous resources, such as modification by alkylating and oxidative agents, spontaneous decomposition products and even absent bases (Memisoglu and Samson, 2000; Seeberg et al., 1995). Base excison repair is initiated through the recognition and removal of damaged bases by DNA glycosylase enzymes. DNA glycosylases remove a variety of damaged bases by cleavage of the N-glycosylic bonds between the bases and the deoxyribose moieties of the nucleotide residues (Krokan et al., 1997). Cocrystal structures of several glycosylases binding with DNA show that the substrate base flips out of the sharply bent DNA helix and the minor groove is widened to be accessed by the glycosylases (McCullough et al., 1999). To complete the repair after glycosylase action, the apurinic/apyrimidinic (AP) site is further processed by an incision step, DNA synthesis, an excision step, and DNA ligation via two alternative pathways, namely short-patch BER and long-patch BER. Both short-patch BER (1-nucleotide patch size) and long-patch BER (2-6-nucleotide patch size) pathways need AP endonuclease to generate a 3'-hydroxyl group but require different sets of enzymes for DNA synthesis and ligation (Fortini et al., 1999; Klungland and Lindahl, 1997; Wilson and Thompson, 1997).

BER is more versatile than other DNA repair mechanisms since it is initiated by at least eight different DNA glycosylases and followed at least two different paths downstream. Lots of research underscore that BER is an important pathway for the avoidance of mutagenic and lethal genetic events. Mutants defective in the AP endonucleases have an increased spontaneous mutation rate and sensitivity to alkylating

and oxidative agents (Xiao and Samson, 1993). In various DNA glycosylase gene deletion mutants, the increased cellular sensitivities to alkylating agents, oxidative agents or other endogenous damage has been reported (Seeberg et al., 1995).

1.2.2. Nucleotide excision repair pathway

The repair of many DNA lesions, including UV or UV-mimetic agents induced damage and bulky chemical adducts requires a functional nucleotide excision repair (NER) pathway. The common feature between these DNA damaging agents is that they all cause helix-distorting lethal lesions.

Nucelotide excison repair begins with the recognition of helical distortion lesions, and then the removal of a 24-32 base oligonucleotide from the strand containing the lesion. The resulting single strand gap is filled in by DNA polymerase and followed by ligation (Friedberg et al., 1995). There are two classes of NER. One is transcription-coupled NER, another is global genome NER. Studies carried out with mammalian cells demonstrate that NER occurs preferentially in actively transcribed genes (Bohr et al., 1985; Madhani et al., 1986). A similar phenomenon has been demonstrated in *E. coli* (Mellon and Hanawalt, 1989) and *S. cerevisiae* (Smerdon and Thoma, 1990; Sweder and Hanawalt, 1992). This preferential repair of the active gene is accounted for by the faster repair of the transcribed strand. The specialized strand directed form of NER is designated transcription-coupled repair NER (TCR). In contrast, NER also can recognize and repair lesions throughout the genome, designated global genomic repair pathway (GGR). GGR is a random process that occurs slowly (Balajee and Bohr, 2000).

The importance of the NER pathway is demonstrated in the association between several human diseases and syndromes and defects in proteins in this pathway. Examples include Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). Patients with XP are characterized by extreme photosensitivity, mental retardation and abnormal pigmentation. They develop skin cancer at an early age and are over 1000-fold more sensitive towards developing cancer in the skin exposed to the sun (van Steeg and Kraemer, 1999). The genes implicated in the pathogenesis of XP groups (A through G) encode proteins that are involved in damage recognition and incision steps of NER.

1.2.3. Mismatch repair pathway

As mentioned above, mispaired bases in DNA can arise by replication errors, spontaneous or induced base modifications, and during recombination. The major pathway for correction of mismatches arising during replication is the MutHLS pathway of *Escherichia coli* (*E. coli*) and related pathways in other organisms. MutS initiates repair by binding to the mismatch, and activates together with MutL the MutH endonuclease, which incises at hemimethylated dam sites and thereby mediates strand discrimination (Modrich and Lahue, 1996). Multiple MutS and MutL homologues exist in eukaryotes, which play different roles in the Mismatch repair (MMR) pathway or in recombination. No MutH homologues have been identified in eukaryotes, suggesting that strand discrimination is different from *E. coli* (Kolodner and Marsischky, 1999). Repair can be initiated by the heterodimers MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ). MLH1-PMS1 (MutLα) is the major MutL homologous heterodimer. Some,

but not all, eukaryotes have additional MutL homologues, which all form a heterodimer with MLH1 and play a minor role in MMR. Additional factors with a possible function in eukaryotic MMR are PCNA, EXO1, and the DNA polymerases δ and ε. Mismatch repair independent pathways or factors that can process some types of mismatches in DNA are NER, some BER glycosylases, and the flap endonuclease FEN-1. A pathway has been identified in *Saccharomyces cerevisiae* (*S. cerevisiae*) and human that corrects loops with about 16 to several hundreds of unpaired nucleotides. Such large loops cannot be processed by MMR (Marti et al., 2002).

Heritable mutations in a MMR gene lead to a so-called mutator phenotype with a very high susceptibility of the cell or organism to mutations (Loeb, 1994). It is known that hereditary nonpolyposis colorectal cancer (HNPCC) is caused by defects in the MMR gene *hMSH2* or *hMLH1* (Buermeyer et al., 1999).

1.2.4. Postreplication repair pathway

The postreplication repair pathway enables the completion of DNA replication in the presence of DNA polymerase blocking lesions. Actually, these lesions are not removed, so the postreplication repair pathway is a DNA damage tolerance pathway. There are two sub-pathways in the postreplication repair pathway. One is the error-free pathway, and another is the error-prone pathway. The exact molecular events in postreplication repair are largely unknown.

Lots of non-essential DNA polymerases have been discovered recently, such as UmuC and DinB in *E. coli,* Rev3-Rev7 (Polζ), Rev1 and Rad30 (Polη) in yeast, hRev3, hRev7, Polη, Polκ and Polι in human (Baynton and Fuchs, 2000; Goodman and Tippin,

2000). These DNA polymerases are largely mutagenic and have abilities to replicate over DNA lesions that block regular replicative DNA polymerases with the expense of an increased mutation rate.

Error- free sub-pathway in yeast is currently known to require Rad6, Rad18, Mms2, Ubc13, Rad5, Pol30 (PCNA), and Pol3 (Polδ) (Broomfield et al., 2001). Within the error-free sub-pathway, genetic studies are further subdivided it into two arms, the Rad5 branch and the Pol30 (PCNA) branch. Both of these branches are thought to be under the control of Mms2 and Ubc13, and these in turn are under the control of Rad6 and Rad18 (Xiao et al., 2000).

1.2.5. Recombination repair pathway

Recombination can be initiated by single-strand or double-strand DNA breaks. SSBs may result during DNA replication or during repair, after IR or UV irradiation or alkylation or cross-linking of DNA bases, or from intermediates of type I topoisomerase. SSB is often changed to DSB in vivo. Double strand breaks can appear as a consequence of IR, by mechanical stress, by endonucleases or by replication of a single-stranded nicked chromosome (Paques and Haber, 1999). Double strand breaks can not be repaired by other DNA repair pathways such as BER, MMR and NER. It only can be repaired by the recombination repair pathway.

Double strand break repair events are classified into two major categories. Homologous recombination events of several types are characterized by the need for the damaged DNA strands to base pair with a homologous partner, where the extent of interaction generally involves hundreds of nearly perfectly matched base pairs. In

contrast, nonhomologous repair events can join ends of DNA with no complementary base pairs at the junction, although in general it turns out that most of these events make use of a very small number of homologous base pairs, known as microhomology (Paques and Haber, 1999).

1.2.5.1. Homologous recombination repair (HRR) pathway

Most of the knowledge about the homologous recombination repair pathway has been obtained from research in bacteria, phages and yeast, where homologous repair events occur at significantly higher frequencies than non-homologous events. There are four major mechanisms of HRR: DSB repair model of Szostak, synthesis-dependent strand annealing (SDSA), break-induced replication (BIR), and single-strand annealing (SSA). The fact that the frequency of HRR in yeast is directly related to the appearance of DSB leads to the conclusion that most, if not all, recombination events are induced by DSB (Wu and Lichten, 1994). To complete the picture of HRR, an earlier HRR model, the Meselson-Radding model which is initiated by an SSB, is also introduced here.

1.2.5.1.1. DSB repair model of Szostak

Gene conversion is defined as a nonreciprocal transfer of genetic information from one molecule to its homologue (Paques and Haber, 1999). The best paradigm for gene conversion is found in meiosis. Because gene conversion is strongly associated with crossovers, molecular models are designed to account for this fact, culminating in the DSB repair model first proposed by Resnick and Martin (Resnick and Martin, 1976) and later elaborated by Szostak and coworkers (Szostak et al., 1983).

After DSB induction, the ends are 5'-3' exonucleolytically resected to produce long 3' single-stranded tails, which are often about 600 nucleotides (Sun et al., 1991). The free end invades an intact homologous template and base pairs with the complementary sequence. The 3' ends of the invading strands can then act as primers for the initiation of new DNA synthesis. This process would lead to the formation of two four-stranded branched structures, known as a Holliday junction (Holliday, 1964) . The Holliday junction is not fixed in space, and it can branch migrate to enlarge the heteroduplex region. The Holliday junction can be cleaved by a resolvase through cutting either the two noncrossed strands or the two crossed strands to form crossover or noncrossover products (Fig. 1-1).

1.2.5.1.2 Synthesis-dependent strand-annealing (SDSA)

Since many mitotic gene conversions are not associated with crossing over, a second model is proposed which is designated as SDSA (Hastings, 1988; McGill et al., 1989; Nasmyth, 1982; Thaler and Stahl, 1988).

The basic feature of SDSA is that the newly synthesized DNA strands are displaced from the template and returned to the broken molecule, allowing the two newly synthesized strands to anneal to each other (Paques and Haber, 1999). The annealing of two newly synthesized strands is either because topoisomerases or helicases dismantle the replication structure actively (McGill et al., 1989), or because the replication "bubble" remains small, with the newly synthesized strand being continuously unwound from its template (Formosa and Alberts, 1986). Unlike the DSB repair model, repair

synthesis in the SDSA model is conservative, namely all newly synthesized sequences are on the same molecule, rather than semi-conservative (Fig. 1-2).

1.2.5.1.3 Break-induced replication (BIR)

A common view of gene conversion is that it involves short-patch events. However, sometimes very long conversion tracks are observed (Esposito, 1978; Golin and Falco, 1988; Voelkel-Meiman and Roeder, 1990). The central feature of the BIR pathway is that only one DSB end invades the homologue or sister chromatid and initiates both leading and lagging strand synthesis in a true replication fork (Chen and Kolodner, 1999; Kogoma, 1996; Kogoma, 1997). Once BIR starts, it can proceed to the chromosome end or be converted into gap repair if the second end of the DSB becomes involved. Break-induced replication may be a biologically important pathway for the repair of chromosome ends. A chromosome that has lost a telomere has a single DSB end because the distal, acentric fragment is lost so that no second end can participate in a gene conversion or SDSA event. Break-induced replication accounts for the recombination-dependent maintenance of telomeres in the telomerase deficient cells (Paques and Haber, 1999; Pfeiffer et al., 2000) (Fig. 1-3).

1.2.5.1.4. Single-strand annealing (SSA)

When a DSB occurs between two flanking homologous regions, repair of the broken chromosome is very efficient and results in a deletion containing a single copy of the repeat and the intervening sequence. A mechanism designated single-stranded annealing (SSA) was first suggested by Lin et al. to explain this event in mammalian cells (Lin et al., 1984; Lin et al., 1985). Single-strand annealing is initiated by the resection of the

ends of the DSB by a 5'-3' exonuclease to produce a long 3' sing-stranded tails, so that the substantial homologous sequences flanking the break in the complementary strands are exposed and can undergo strand annealing. After excision of the nonhomolgous 3' ends and new DNA synthesis, ligation restores two continuous strands (Fig. 1-4). In yeast, SSA is nearly 100% efficient if the homologous regions flanking the DSB are at least 400 bp (Sugawara and Haber, 1992), and repair is efficient even if the repeats are separated by as far as 15 kb.

1.2.5.1.5. Meselson-Radding model

This model was initially proposed by Meselson and Radding (Meselson and Radding, 1975). It is very similar to the DSB repair model of Szostak**,** but the homologous recombination is initiated by a single-stranded break (SSB) instead of a double stranded break (DSB) in this model. The 3'-end of the SSB induced in one DNA molecule is used as a primer for displacement synthesis. The displaced 5' single-strand pairs with the complementary sequence in the homologue or sister chromatid and induces an SSB in the latter which initiates a reciprocal strand exchange to create a joint molecule with a single Holliday junction that can branch migrate. As with the Szostak's model, either a crossover or a a non-crossover product result after resolution of the Holliday junction.

In an *in vitro* system, the bacteriophage T4 uvsX protein (a RecA-like strand transferase) and part of the T4 DNA polymerase holoenzyme efficiently mediates pairing between nicked double-stranded circular and linear duplex DNAs, thereby demonstrating the feasibility of a key step in the Meselson-Radding model (Kodadek and Wong, 1990).

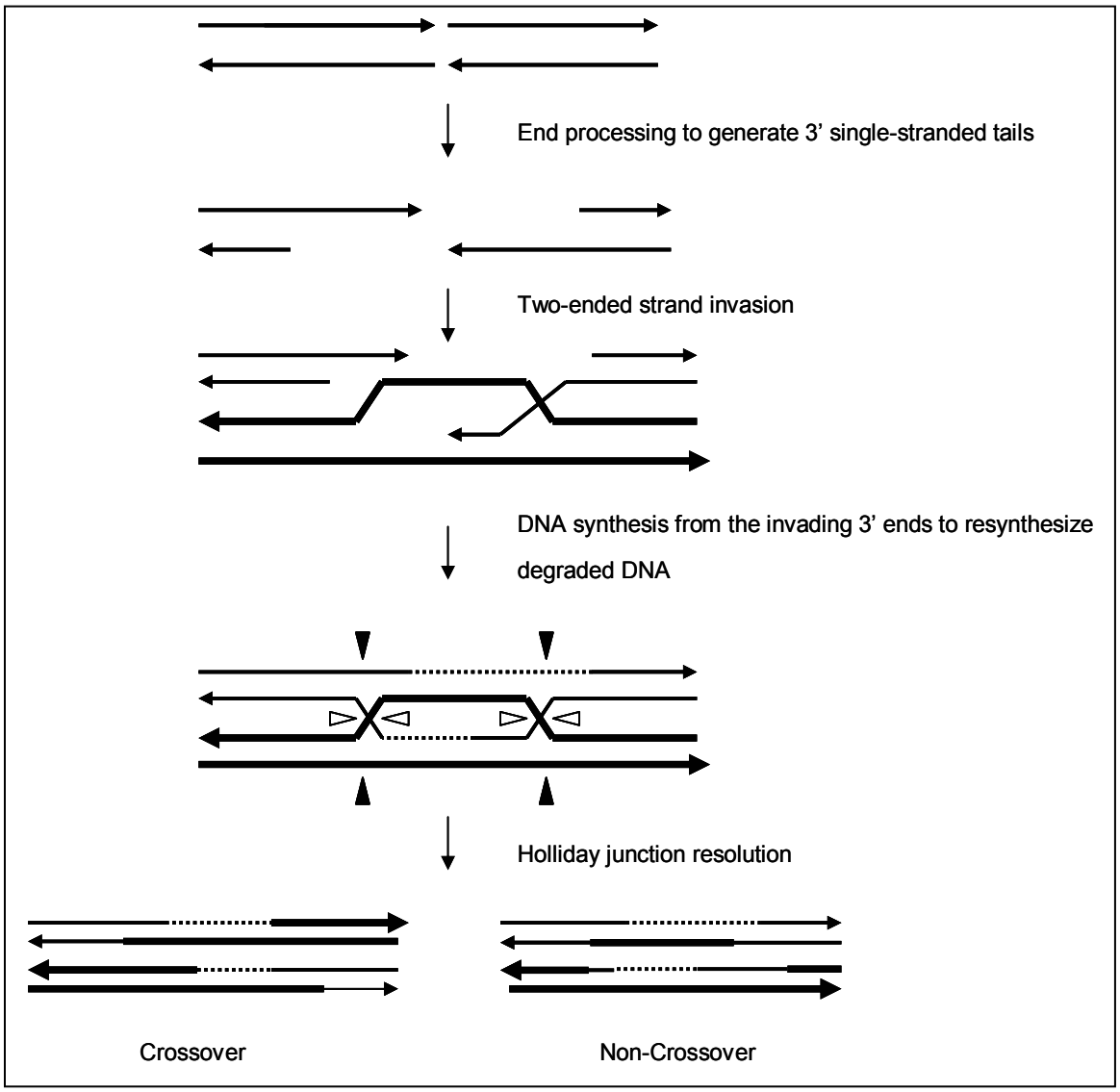

Figure 1-1. DSB repair model of Szostak. In this model, the ends are processed to yield 3'single-stranded tails. The 3' ends invade the homologous duplex, priming DNA synthesis. After ligation, two Holliday junctions are formed, which can subsequently be resolved by endonucleolytic cleavage to generate crossover or noncrossover products. Adapted from Symington (2002).

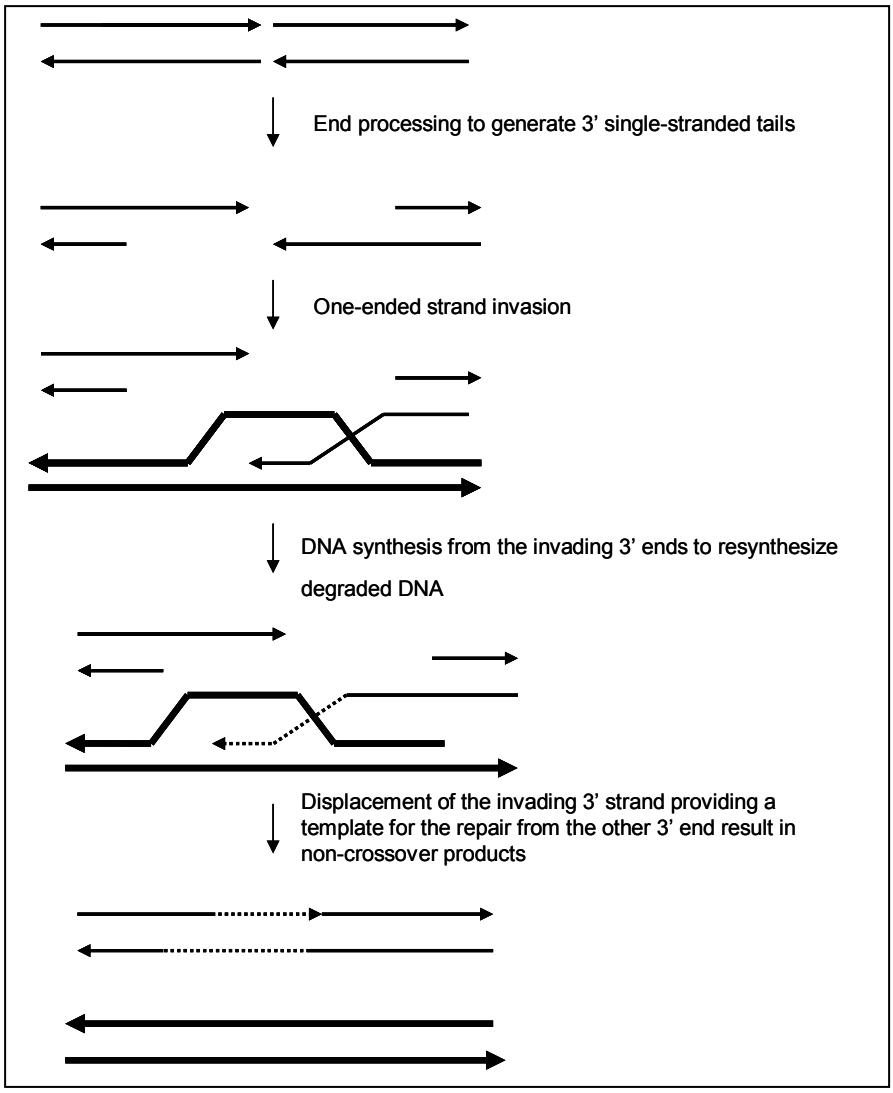

Figure 1-2. The Synthesis-dependent strand-annealing (SDSA) model. In the SDSA model, the ends are processed to yield 3' single-stranded tails, one of which invades the homologous duplex, priming DNA synthesis. The displacement loop (D-loop) formed by strand invasion could be extended by DNA synthesis or could migrate with the newly synthesized DNA. After displacement from the donor duplex, the nascent strand pairs with the other 3' single-stranded tail and DNA synthesis completes repair. Adapted from Symington (2002).

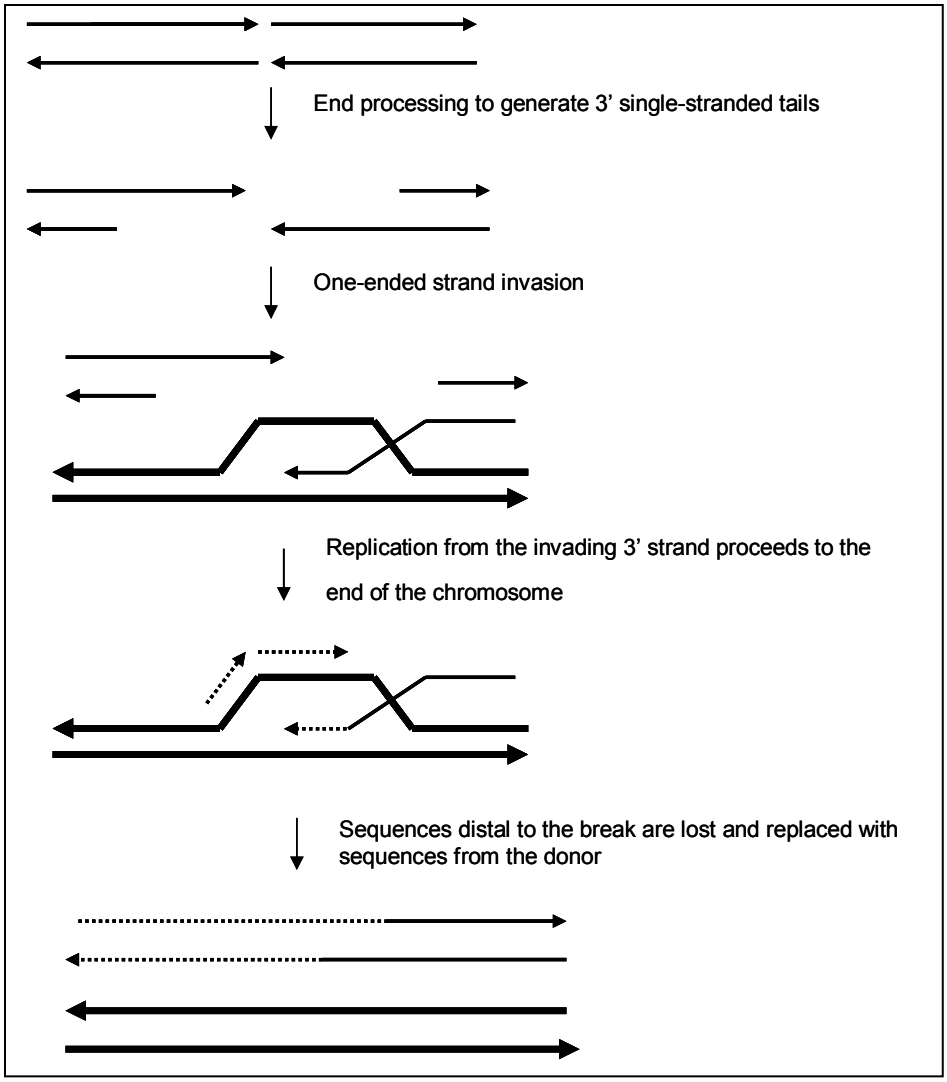

Figure 1-3. The Break-induced replication (BIR) model. The initial steps in the BIR model are the same as in the SDSA model, but DNA synthesis from the invading strand continues to the end of the DNA molecule. So the sequences distal to the break are lost and replaced with sequence from the donor DNA. Adapted from Symington (Symington, 2002).

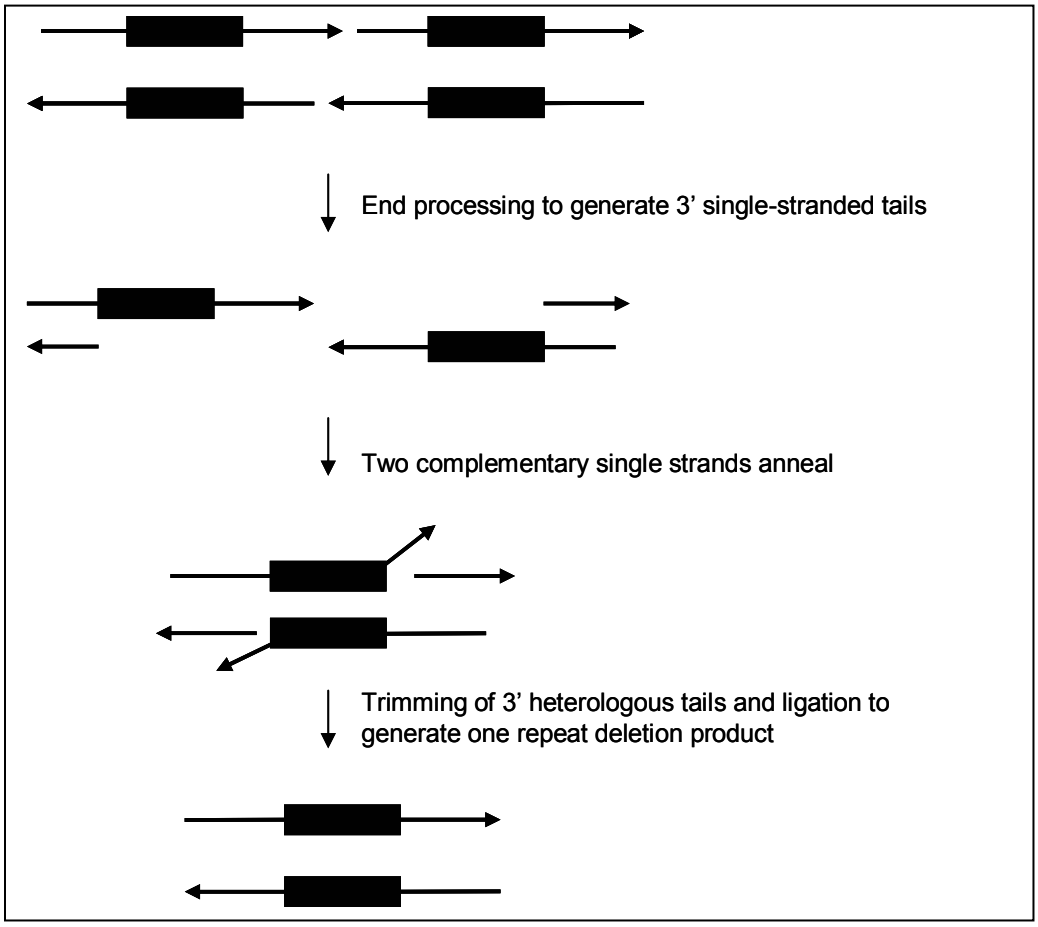

Figure 1-4. The Single-strand annealing (SSA) model. In the SSA model, a DSB made between direct repeats is subject to resection to generate 3' single-stranded tails. When complementary sequences are revealed due to extensive resection, the single-stranded DNA anneals, resulting in deletion of one of the repeats and the intervening DNA. The 3' tails are endonucleolytically removed, and the nicks are ligated. Adapted from Symington (2002).

So far, this is the only experiment to support this model. However, there are no experiments rule out this model.

1.2.5.2 Genes involved in homologous recombination repair pathway in *S. cerevisiae*

Genes important for the repair of DSB were identified primarily as their corresponding mutants are sensitive to X rays but not to UV irradiation. These genes were classified as the *RAD52* epistasis group. Currently 8 genes in this group are thought to play important roles in homologous recombination repair, namely *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *MRE11* and *XRS2* (Table 1-1). All these genes have homologs in vertebrate. The proteins encoded by these genes can be classified into two families according to their biochemical properties. One family participates in the strand transfer reaction. It contains Rad51, Rad52, Rad54, Rad55 and Rad57, whereas another family including Mre11, Rad50 and Xrs2 is responsible for nuclease activity.

1.2.5.2.1 *RAD51*

RAD51 encodes a 43-kD protein with 30% identity to bacterial RecA, which is the pivotal protein involved in all homologous recombination events in *E. coli* (Kowalczykowski et al., 1994; Shinohara et al., 1992). Unlike *RecA* in *E. coli*, *RAD51* is not indispensable for homologous recombination in yeast. A number of HR events can occur without *RAD51*, including SDSA, SSA and the maintenance of telomeres by BIR in the absence of telomerase (Le et al., 1999). Yeast *rad51* null mutants are viable but show high level sensitivity to IR and meiotic inviability. In vertebrates, deletion of *RAD51* is lethal.

Table 1-1. The genes involved in homologous recombination repair pathway.

Models	Involved genes
DSB repair model of Szostak	*RAD52*, *RAD51*, *RAD54*, *RAD55*, *RAD55*, *RAD57*, *RAD50/MRE11/XRS2*
SDSA	*RAD52*, *RAD51* (?), *RAD50/MRE11/XRS2* (?)
BIR	*RAD52*, *RAD50/MRE11/XRS2* (?)
SSA	*RAD52*, *RAD50/MRE11/XRS2* (?), *RAD1/RAD10*

Note : (?) represents the gene which is hypothesized to be required in the corresponding model without direct experimental proof.

Like RecA, Rad51 has Walker A and B motifs required for ATP binding and/ or hydrolysis (Aboussekhra et al., 1992; Shinohara et al., 1992). Purified Rad51 forms right-handed helical filaments on double-stranded DNA with structural similarity to those formed by RecA (Ogawa et al., 1993), and Rad51 binds with higher affinity to DNA duplex with single-stranded tails than duplex or single-straded oligonucleotides (Mazin et al., 2000). Formation of filaments on ssDNA is enhanced by the presence of the yeast single-stranded binding complex RPA (Sung, 1994; Sung and Robberson, 1995), which may remove secondary structure from ssDNA to allow the formation of a continuous Rad51 nucleoprotein filament. However, *in vitro*, it results in a severe reduction in strand exchange products if RPA is added simultaneously with or prior to Rad51. The inhibition can be overcome by addition of Rad52 or Rad55-Rad57 heterodimer to the reaction mix (Shinohara and Ogawa, 1998; Sung, 1997a; Sung, 1997b). Once the Rad51 nucleoprotein filament is assembled , it can catalyze an ATP-dependent strand exchange between a single-stranded circular molecule and a homologous linear duplex (Namsaraev and Berg, 1997; Sung, 1994; Sung and Robberson, 1995).

1.2.5.2.2 ***RAD52***

The *RAD52* gene encodes a strand annealing protein of 471 amino acids. Rad52 does not show obvious homology to any of the known recombination proteins in bacteria, and therefore appears to be unique to eukaryotes. In contrast to *RAD51*, *RAD52* is required for virtually all homologous recombination events. Deletion of *RAD52* in budding yeast is not lethal, but results in severe defects in homology-dependent DNA recombination repair and meiosis. It is the only X-ray sensitivity gene required for SSA

(Sugawara and Haber, 1992). Break-induced replication also depends on *RAD52,* but not on *RAD51*.

Both yeast and human Rad52 are self interacting and form a ring structure (Ranatunga et al., 2001; Shinohara et al., 1998; Stasiak et al., 2000). Human Rad52 was observed to form heptameric rings with a strong pinwheel appearance and a central channel (Stasiak et al., 2000). DNA might be bound within the central channel. The purified Rad52 binds preferentially to ssDNA and promotes annealing of complementary ssDNA (Mortensen et al., 1996). Rad52-promoted annealing of long molecules is stimulated by RPA, whereas Rad52 efficiently anneals oligonucleotides in the absence of RPA (Mortensen et al., 1996; Shinohara et al., 1998; Sugiyama et al., 1998).

Genetic studies with yeast suggest a *RAD52*-dependent, *RAD51*-independent pathway for strand invasion, but no biochemical data show that Rad52 can catalyze the invasion of a single strand into a double-stranded molecule. Besides self interaction, Rad52 has been demonstrated to interact with Rad51 both biochemically and genetically (Donovan et al., 1994; Milne and Weaver, 1993; Shen et al., 1996). The Rad51 interaction domain of Rad52 is necessary for overcoming the RPA inhibition to strand exchange *in vitro*, consistent with the model that the mediator function of Rad52 requires interaction between Rad52 and Rad51 (Krejci et al., 2001; Shinohara and Ogawa, 1998). Meanwhile, Rad52 has been found to physically interact with the 30KDa subunit of RPA (Shinohara et al., 1998). Thus, Rad52 seem to play a highly specific role in the homologous pairing and strand exchange reaction, mediating a productive interaction between Rad51 and RPA (Sung, 1997a). Because of this function, Rad52 has been called a "mediator" of DNA strand exchange. It is still unknown how Rad52 exerts the

mediator function, but it is supposed to target Rad51 to ssDNA, which can then act as the nucleation sites for filament growth.

1.2.5.2.3 ***RAD54***

RAD54 encodes a protein which appears to be a member of a diverse family of chromatin-remodeling proteins including the transcription factors Swi2/Snf2 (Emery et al., 1991). These proteins have the sequence motifs characteristic of DNA helicases, but no helicase activity has been demonstrated in any of these proteins. As with other *RAD52* group genes, *RAD54* is not essential for viability in yeast but required for IR resistance.

Rad54 possesses dsDNA-dependent ATPase activity and promotes a conformational change of closed-circular duplex due to the creation of positive and negative writhe (Petukhova et al., 1998; Swagemakers et al., 1998; Tan et al., 1999; Van Komen et al., 2000). Rad54 physically interacts with Rad51 (Clever et al., 1997; Jiang et al., 1996). The first DNA intermediate predicted in the DNA DSB repair model for recombination is a D-loop structure formed between the initiating ssDNA tail and its DNA homolog. While Rad51 is incapable of mediating D-loop formation *in vitro*, the inclusion of Rad54 renders D-loop formation possible (Petukhova et al., 1998). However, Rad54 does not have homologous DNA pairing activity by itself. Recent studies show that Rad54 forms a co-complex with Rad51-DNA filament and stabilizes it, which stimulates DNA strand exchange and stabilizes the nascent DNA heteroduplex in later steps (Mazin et al., 2003). D-loop formation by Rad54 and Rad51 occurs with > 100 fold higher efficiency with chromatin relative to naked DNA in the absence of superhelical torsion (Alexiadis and

Kadonaga, 2002), suggesting Rad54 is involved in much more important role to function with chromatin *in vivo* than naked DNA used for experiments *in vitro*. Meanwhile, Rad54 may also have a chromatin remodelling function since many members of the Swi2/Snf2 family of proteins function to remodel chromatin. DNA remodelled by Rad54 becomes more sensitive to the ssDNA-specific nuclease P1, indicating transient strand separation (Van Komen et al., 2000). Rad54 is able to translocate itself along the nucleosomal fiber and generates superhelical torsion with the ATPase activity, thus leading to enhanced nucleosomal DNA accessibility without disrupting nucleosome position (Jaskelioff et al., 2003). All of the biochemical activities of Rad54 except dsDNA binding are dependent on ATP hydrolysis (Petukhova et al., 2000; Solinger et al., 2001).

1.2.5.2.4 ***RAD55* and *RAD57***

RAD55 and *RAD57* are unique among the *RAD52* group members because their null mutants are cold sensitive to IR (Lovett and Mortimer, 1987). *RAD55* and *RAD57* have a tight epistatic relationship, and the proteins encoded by them can form a heterodimer (Hays et al., 1995; Johnson and Symington, 1995; Sung, 1997b).

Rad55 and Rad57 share some limited homology to RecA and Rad51. All of them have a Walker nucleotide binding motif (Lovett, 1994). A mutation in a conserved Walker type A lysine in Rad55 affects recombination, while an analogous mutation in Rad57 has no effect (Johnson and Symington, 1995). Addition of the Rad55-Rad57 complex to a strand exchange reaction effectively overcomes the competition posed by RPA, so the heterodimer may also function as a mediator. Although the Rad55-Rad57

heterodimer has ssDNA binding activity, it does not replace RPA in strand exchange and does not appear to possess strand exchange activity (Sung, 1997a). Since Rad52 has been identified as a mediator as well, it is possible that the Rad55-Rad57 heterodimer and Rad52 act in a different, parallel or overlapping pathway to ensure the assembly of Rad51 nucleoprotein filament occurs efficiently *in vivo*.

1.2.5.2.5 ***RAD50*, *MRE11* and *XRS2***

In budding yeast, null mutations in *RAD50*, *MRE11* or *XRS2* cause similar phenotypes. These mutants show poor vegetative growth, defects in meiosis, and high sensitivity to IR (Ajimura et al., 1993; Game and Mortimer, 1974; Ivanov et al., 1992). As indicated by yeast two-hybrid and coimmunoprecipitation studies, the three proteins function as a complex (Johzuka and Ogawa, 1995; Usui et al., 1998). The Mre11 and Rad50 are highly conserved in all organisms, whereas Xrs2 is weakly conserved and is found only in eukaryotes. Deletion of *MRE11*, *RAD50* or *XRS2* is not lethal in yeast, but they are essential for viability in vertebrates (Luo et al., 1999; Zhu et al., 2001).

RAD50, *MRE11*, and *XRS2* are involved in the nucleolytic processing of DSBs. Mre11 and Rad50 are homologous to SbcD and SbcC respectivly, which are two interacting proteins in bacteria. SbcD has double-stranded exonuclease and single-stranded endonuclease activity (Sharples and Leach, 1995). Yeast Mre11 has ssDNA endonuclease and weak 3' to 5' exonuclease activities (Furuse et al., 1998; Moreau et al., 2001; Paull and Gellert, 1998; Usui et al., 1998). The nuclease activities lies in the N-terminal part of Mre11, because mutations in this region eliminate the nuclease activities *in vitro* and reduce the 5'-3' resection *in vivo* (Furuse et al., 1998; Tsubouchi and Ogawa,

1998; Usui et al., 1998). In contrast, mutations in the C-terminus do not affect the resection of DSB ends (Furuse et al., 1998; Nairz and Klein, 1997).

The 153 KDa Rad50 protein is related to the structural maintenance of chromosomes (SMC) proteins, which have Walker A and B motifs characteristic of NTP binding (Alani et al., 1989). Mutation of the conserved lysine residues within the Walker A box confers a null phenotype in yeast, suggesting the importance of ATP binding and/or hydrolysis (Alani et al., 1990). Although the DNA binding activity of yeast Rad50 is stimulated by ATP, no ATPase activity has been observed for the purified protein (Raymond and Kleckner, 1993). The endonuclease activities of Mre11 are enhanced markedly by Rad50 in the presence of ATP (Trujillo and Sung, 2001). Although hRad50 has been shown to stimulate the exonuclease activity of human Mre11(Paull and Gellert, 1998), Rad50 of *S. cerevisiae* does not appear to significantly affect the exonuclease activity of *S. cerevisiae* Mre11 (Trujillo and Sung, 2001).

Xrs2 is a 96 KDa protein. In yeast, it interacts with the Rad50-Mre11 complex through the Mre11 subunit (Johzuka and Ogawa, 1995; Usui et al., 1998). The biochemical functions of Xrs2 and its role in modulating the activities of Rad50 and Mre11 is still unknown. The human equivalent of Xrs2, NBS1, is essential for Mre11 phosphorylation in the human complex and enhances the DNA unwinding and endonuclease cleavage activity of fully paired hairpins by hRad50·hMre11 (Dong et al., 1999; Paull and Gellert, 1999).

1.2.5.3. Homology-independent recombination repair pathway

In mammalian cells, most DSB repair occurs through non-homologous recombination rather than homologous recombination. This non-homologous recombination mechanism, also termed non-homologous end joining (NHEJ), is able to rejoin DSB directly. The independence of homologous sequence in NHEJ does not necessarily mean that homology is never involved. On the contrary, whenever short regions of sequence homology, so-called micro-homology patches in the range of 1-10 bp, are available they will most probably be used (Pfeiffer et al., 2000).

NHEJ was first observed in mammalian cells (Pellicer et al., 1980; Perucho et al., 1980) and later also reported in yeast (Orr-Weaver and Szostak, 1983). The simplest model of NHEJ is the ligation of compatible ends. However, NHEJ is also able to rejoin non-complementary ends irrespective of their sequence and structure. This has implications in the mutagenic potential of the DSB repair pathway: (i) the original sequence is only restored if the DSB generates two complementary or blunt ends that can be precisely religated; (ii) if, however, two non-matching ends (for instance after irradiation) arise they first have to be converted into a ligatable structure by enzymatic modification, which often causes base pair substitutions, insertions and/or deletions. Although this will usually lead to small scale mutations at the resulting repair site (junction), the consequences of NHEJ appear to be tolerable on multicellular organisms because the chance that small alterations at break points affect a critical region within an expressed essential gene is low due to the favorable ratio of non-coding to coding DNA (only a small portion of total genomic DNA in a mammalian cell encodes functional gene), and even in the case of such an unlikely event the intact allele may compensate for defective alleles in diploid cells (Pfeiffer et al., 2000).

In budding yeast, efficient NHEJ is only detected when the *RAD52*-dependent HRR pathway is disabled. The NHEJ pathway is dependent on a heterodimer, Ku protein (Doherty and Jackson, 2001; Jones et al., 2001). The Ku protein binds to DNA ends, stabilizing them for processing and religation. Normally, strand breaks induced by ionizing radiations or other agents can not simply religate because of their modified termini. So the Ku protein does not just act by stabilizing the DNA ends, it can also facilitate processing of the ends. The Rad50-Mre11-Xrs2 complex is required for NHEJ (Moore and Haber, 1996; Tsukamoto et al., 1996), and is likely invovled in processing DNA ends. Ligase IV is responsible for the ligation activity (Frank et al., 1998; Grawunder et al., 1998; Wilson et al., 1997). *RAD27* of *S. cerevisiae* encodes a structural and functional homolog of the 5'-3' exonuclease function of *E. coli* DNA polymerase I. Deletion of *RAD27* leads to a reduction in NHEJ events that proceed through a 5' flap intermediate (Wu and Wang, 1999). Furthermore, mutations in *RAD27* are lethal in combination with mutations in *RAD50*, *RAD51* or *RAD52* (Symington, 1998). These results suggest that Rad27 is one of the nucleases involved in end possessing for the NHEJ pathway.

Many of cancer-prone genetic disorders are involved in proteins associated with recombination DNA repair. For example, Ataxia telangiectasia (AT), Nijmegen breakage syndrome (NBS), and an ataxia-like disorder (ATLD) are chromosome instability disorders that are defective in the ataxia telangiectasia mutated (ATM), NBS, and Mre11 genes, respectively. These genes have been proved to play an important role in recombination DNA repair (Nelms et al., 1998; Rotman and Shiloh, 1999).

1.3. Holiday junction resolvase

1.3.1. Formation and resolution of Holiday junction

As mentioned before, homologous DNA recombination repair is initiated by exchange of single strands between two homologous DNA duplexes to form an X-structure known as the Holiday junction. This structure is an intermediate in both homologous and certain site-specific recombination reactions. DNA molecules interlinked in this way have to be separated by structure-specific endonucleases that catalyze dual-strand incision across the point of strand cross-over.

Holliday junctions can also arise at stalled replication forks by reversing the direction of fork progression and annealing of nascent strands. Junction formation entails the annealing of newly synthesized strands in a reaction that is probably catalyzed by a branch-specific helicase (McGlynn and Lloyd, 2000). The Holliday junction can be removed directly by resetting the replication fork, degrading the ends of the extruded duplex, or by endonucleolytic resolution. Resolution of junctions in the last instance generates a DNA break and thus serves to initiate rather than terminate recombination (Seigneur et al., 1998).

1.3.2. The structure of Holliday junction

Although the detailed Holliday junction structure has not yet been totally determined from X-ray or nuclear magnetic resonance studies, a variety of other experimental approaches and molecular modeling studies have provided two major conformations of Holliday junction *in vitro*. Small four-way junction structures that mimic the properties

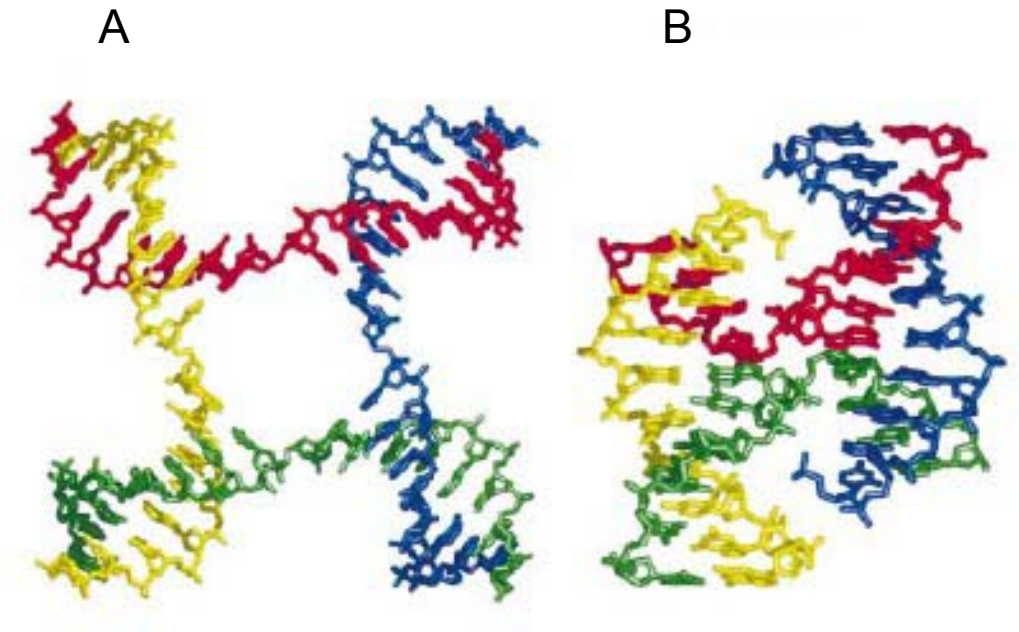

Figure 1-5. Holliday junction structures. Holliday junctions can adopt two alternative conformations in solution (Duckett et al., 1993). A, the square planar junction in the absence of metal ions. B, the stacked-X in the presence of Mg^{2+}. Adapted from Sharples (2001).

of a true Holliday junction have been constructed by annealing partially complementary oligonucleotides. In the absence of metal ions, the charge on the phosphate backbone causes the four duplex arms to adopt a square planar configuration with approximately four fold symmetry (Hargreaves et al., 1998). When divalent metal ions are present,the junction is arranged in a two fold symmetrical stacked-X conformation (Eichman et al., 2000). In this form, the duplex arms stack on each other, resulting in two continuous strands of opposite polarity traversing the outer edge of the junction core, whereas the second pair of strands is exchanged (Fig. 1-5). In both forms, the faces of the junction are different, exhibiting minor groove characteristics on one side and major groove features on the other. The relevance of these junction configurations *in vivo* is unclear because the Holliday structure will be constrained within large stretches of DNA and coated with an assortment of nucleic acid-binding proteins. In any case, regardless of its initial configuration, the arrangement of junction arms is ultimately determined by the specific architecture and requirements of the Holliday junction-binding protein (Sharples, 2001).

1.3.3. Endonucleases that catalyze Holliday junction resolution

Holliday junction resolvases are characterized by their capacity to recognize four-way DNA structures and introduce paired incisions located symmetrically across the point of strand exchange (White et al., 1997). Normally, resolvases are dimeric, facilitating the positioning of two active sites for simultaneous, or near-simultaneous, strand scission (Giraud-Panis and Lilley, 1997). Holliday junction resolvases are highly selective for specific structures, and most impose a particular

conformation on the junction upon binding, presumably to allow placement of the scissile bond in the vicinity of the catalytic site.

1.3.3.1 The RuvABC complex in *E. coli*

The RuvAB and RuvC proteins have been shown to catalyze the resolution of Holliday junctions in *E. coli*. A tetramer of 22-KDa RuvA protein forms a grooved platform on which the Holliday junction is held in a square planar configuration (Hargreaves et al., 1998; Nishino et al., 1998; Rafferty et al., 1996). RuvB is a helicase that assembles as a hexameric ring on opposite arms of the junction via contacts with RuvA. Branch migration of the Holliday junction is achieved by the RuvB helicase motor drawing DNA through the complex, with each duplex arm rotating within channels on the surface of RuvA (Hargreaves et al., 1998; Parsons et al., 1995).

RuvC of *E. coli* was initially isolated as a junction resolvase activity from purified cell extracts that was found to be missing in samples recovered from *ruvC* mutants (Connolly et al., 1991). The dimeric RuvC endonuclease resolves the Holliday junction into duplex products by the introduction of symmetrically related nicks in two of the four DNA strands. RuvC exhibits a marked preference for Holliday junctions and is unable to recognize or cleave other branched DNA junctions (Dunderdale et al., 1991; Iwasaki et al., 1991). This junction selectivity may result, in part, from its preference for resolving at a tetra-nucleotide sequence with the consensus 5'-(A/T) TT (G/C)-3' (Eggleston and West, 2000). RuvC is thought to scan the junction for its preferred target sequence since the junction is drawn through the RuvABC complex. The folding of the duplex arms encountered may be imperative for the efficient resolution.

1.3.3.2 *E. coli* RusA

The recombination and DNA repair defects associated with mutations in *ruv* can be suppressed by insertion of IS*2* or IS*10* elements upstream of the *rusA* coding region, which activate transcription of the normally silent gene on the cryptic prophage DLP12 (Mahdi et al., 1996; Mandal et al., 1993). The 14-kDa RusA protein is a homodimeric Holliday junction-specific endonuclease (Sharples et al., 1994). It is sequence specific like the RuvC resolvase, although it prefers to cut at the 5' end of a CC dinucleotide. RusA displays less structure specificity than RuvC and can bind a variety of branched DNA structures, but it will only cleave four-way junction DNA and exhibits the preference for cutting 5' of a CC dinucleotide (Chan et al., 1998; Giraud-Panis and Lilley, 1998). The global structure of the Holliday junction is affected by RusA binding, although the precise nature of modifications is uncertain. After binding with RusA, the arms of the junction are equidistant and the junction may be held in a tetrahedral conformation (Chan et al., 1998; Giraud-Panis and Lilley, 1998). Three aspartic acids (D70, D72 and D91) in the conserved C-terminal domain of *E. coli* RusA are essential for catalysis and likely constitute the metal binding site (Bolt et al., 1999). Conserved basic residues are also important for junction binding (R69) and junction cleavage (K76) (Bolt et al., 1999).

1.3.3.3 Yeast Cce1

The *S. cerevisiae CCE1* (cruciform- cutting endonuclease) encodes a 41 kDa endonuclease. The *cce1* null mutant has no obvious growth defect and despite the ability of Cce1 to cleave Holliday junction analogs, the mutant shows no defect in meiotic or

mitotic recombination. A second cruciform cutting activity was detected in extracts from a *cce1* null mutant, indicating that yeast has at least two such enzymes (Kleff et al., 1992). Later, Cce1 was found to be exported to the mitochondria, where it participates in the resolution of recombination intermediates (Lockshon et al., 1995). A protein Ydc1 from *Schizosaccharomyces pombe* (*S. pombe*) shares 28% identity with Cce1, exhibits similar biochemical properties and complements the DNA repair defect associated with *E. coli ruvC* mutation. Like Cce1, Ydc1 is also thought to be catalytically active only in mitochondria (Oram et al., 1998; Whitby and Dixon, 1997).

Although Cce1 has similar properties to *E. coli* RuvC, there are a few notable differences in terms of sequence specificity and the junction conformation imposed upon binding. Cce1 prefers to cleave a tetranucleotide with the consensus sequence 5'-ACT↓A-3', where the two central pyrimidines has been shown to be most important (Schofield et al., 1998). Although both RuvC and Cce1 hold the junction in an open configuration, Cce1 imposes a fully open square planar form (White and Lilley, 1997). Both resolvases disrupt base pairing at the point of strand cross-over (Bennett and West, 1995; Declais and Lilley, 2000; White and Lilley, 1997). Hence, although these proteins are structurally and functionally equivalent, the subtle changes in architecture have important consequences for junction conformation and sequence recognition. It is reasonable to assume that these two processes are intrinsically linked (Sharples, 2001).

1.3.3.4 Resolvases in mammalian cells

Much less is known about Holliday junction resolution in mammalian cells. Resolvase activities have been detected in mammalian cell-free extracts (Chen et al.,

2001; Elborough and West, 1990; Hyde et al., 1994). However, the resolvase itself remains unidentified. The cell-free extract was shown to fit the bacterial RuvABC resolvasome paradigm by its functional association with an ATP-dependent branch migration activity (Constantinou et al., 2001). Unlike the yeast resolvases Cce1 and Ydc1, this activity was not compartmentalized to the mitochondria.

1.4. Cloning and characterization of *MMS4* (*EME1*) and *MUS81*

1.4.1. Cloning of *MMS4* (*EME1*) and *MUS81*

The *MMS4* (methyl methanesulfonate sensitive) gene was first isolated by functional complementation of the methyl methanesulfonate (MMS)-sensitive phenotype in the *mms4-1* strain. Deletion of *MMS4* causes yeast cells to exhibit an increased sensitivity to MMS, MNNG (*N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine), DMS (Dimethyl sulfonate) and EMS (ethyl methanesulfonate), but not to ionizing radiations. *MMS4* encodes a 691-amino acid, 78.7-KDa protein (Fig. 1-6). Several putative functional domains suggest that it may be a nuclear protein capable of interacting with other proteins (Xiao et al., 1998).

In budding yeast, *MUS81* (MMS, UV sensitive) was identified in a yeast 2-hybrid screen using Rad54 as a bait. Deletion of *MUS81* causes a recessive MMS- and UV-sensitive phenotype. However, *mus81Δ* cells are not sensitive to γ-radiation or double-strand breaks induced by an HO endonuclease. *MUS81* encodes a 632-amino acid protein, which has two helix-hairpin-helix motifs and a XPF endonulease motif (Interthal and Heyer, 2000) (Fig 1-7).

Budding yeast Mus81 and Mms4 were also identified as synthetic lethal mutations with the deletion of Sgs1 helicase (Mullen et al., 2001). Meanwhile, Mms4 and Mus81 were coimmunoprecipitate from cell extract, suggesting that they form a complex *in vivo*. Later, Mms4 and Mus81 were shown to form a heterodimer in a 1:1 molar ratio (Kaliraman et al., 2001). Epistatic analysis also indicates that *MMS4* and *MUS81* function in the same DNA repair pathway (Mullen et al., 2001).

In fission yeast, SpMus81 (*S. pombe* Mus81) was found through its interaction with Cds1 (Rad53 orthologue in fission yeast) (Boddy et al., 2000). The SpMus81 is a 572 amino acid, 64.5-KDa protein, and shows significant homology to ScMu81 (*S. cerevisiae* Mus81) (overall identity 27%, overall similarity 38%) (Interthal and Heyer, 2000). SpMus81 was found in a complex with another protein named Eme1 (essential meiotic endonuclease) (Boddy et al., 2001). Unlike Mus81, which shows significant evolutionary conservation, Eme1 is only weakly homologous to Mms4. In addition, Eme1 has a weak but statistically significant sequence homolog in *Neurospora crassa* (Boddy et al., 2001). The extreme sequence divergence of Mms4/Eme1 may explain why related genes have not been detected in the genome database of more complex eukaryotes by standard search methods. Recently, human Mms4 was identified based on a comparison search of three-dimensional (3-D) structure in the human database (Ogrunc and Sancar, 2003). The human *MMS4* gene encodes a protein of 583 amino acids with limited amino acid sequence homology to the budding yeast Mms4 and Eme1.

1.4.2. Biochemical properties of the Mus81-Mms4/Eme1 complex

As mentioned above, Mus81 has two motifs found in the XPF endonuclease superfamily (Boddy et al., 2001; Interthal and Heyer, 2000). One is the XPF endonuclease motif, which contains the highly conserved V/IERKX_3D amino acid sequence (Aravind et al., 1999; Enzlin and Scharer, 2002). The endonuclease activity is abolished when consensus residues in this motif are mutated. The other motif is the helix-hairpin-helix motif. This motif is found in XPF and other proteins involved in DNA metabolism (Doherty et al., 1996). It is thought to allow nonspecific DNA binding via the phosphate backbone (Fig. 1-7).

ScRad1-Rad10 (XPF-ERCC1 in human) was first known to cleave at the 5' side of the UV-induced photoproducts and bulky lesions during nucleotide excision repair (Bardwell et al., 1994). It was also thought to act as a 3'flap endonuclease (Paques and Haber, 1999) that can remove a 3'-ended ssDNA nonhomologous tail from intermediates of recombination, such as those formed during strand invasion if the terminal portion of ssDNA does not match its template, or structure in SSA (Haber and Heyer, 2001) (Fig. 1-8B). The amino acid sequence similarity of Mms4-Mus81 to Rad1-Rad10 suggested that it might posses a nuclease activity. This hypothesis has been proven (Kaliraman et al., 2001; Whitby et al., 2002). Results from the incubation of the Mus81-Mms4 complex with a variety of ^{32}P-labeled DNA structures show that the complex specifically cleaves branch substrates consisting of duplex DNA with noncomplementary tails (Y-form) (Fig. 1-8A). Compared with the activities of Rad1-Rad10, the activities of ScMus81-Mms4 are distinctly different. Rad1-Rad10 cuts the 3' end of double-stranded DNA with two single –strand extensions that are not homologous to each other. Relative to the simple Y-form, both Rad1-Rad10 and XPF-ERCC1 are less active on duplex DNA with a 3'

ssDNA protrusion or branch (de Laat et al., 1998; Rodriguez et al., 1996). In contrast, the ScMus81-Mms4 endonuclease activity is more active on this substrate relative to the simple Y-form. When the 3' protrusion is a duplex, as in duplex DNA with a 5' ssDNA branch, Mus81-Mms4 fails to cleave, suggesting that the duplex 3' arm is not recognized as a substrate (Fig. 1-8). The fact that Mms4-Mus81 is able to completely cleave the duplex Y-form, a replication fork (RF) substrate, suggests that the enzyme recognizes the single- or double-stranded nature of the uncleaved 5' arm with dsDNA acting as a positive effector. Recent studies demonstrated the Mus81-Mms4 cleavage site is determined not by the branch point, like Rad1-Rad10, but by the 5' end of the DNA strand at the flap junction (Bastin-Shanower et al., 2003). Taken together with the fact that *mms4* and *mus81* phenotypes are distinct from *rad1* and *rad10* phenotypes, it suggests that the Mus81-Mms4 complex might be essential for the removal of nonhomologous tails formed during recombination if there were a heterology at the end of the DSB (Kaliraman et al., 2001).

Another hypothesis was proposed through research on the role of *MMS4* in the processing of recombination intermediates during meiosis in budding yeast (de los Santos et al., 2001). The pathway of SDSA initiates with a single end invasion (Fig. 1-2). DNA synthesis extends the length of the invading strand, which then dissociates from the nonsister chromatid and anneals to the resected 3' end on the other side of the break. If extension of the invading strand creates a single-stranded tail that is longer than the resected end on the other side of the break, a 3' "flap" will result after the two strands anneal (Fig. 1-8C). The resulting structure looks identical to the preferred substrate for Mus81-Mms4 *in vitro*. It was proposed that Mus81-Mms4 is required to cleave this flap

so that one side of the break can be repaired by ligation. Failure to remove this flap would result in a broken chromatid (de los Santos et al., 2001) (Fig. 1-8C).

Research on SpMus81-Eme1 presents a rather different biochemical role of the complex (Boddy et al., 2001). In fission yeast, the spores are highly inviable in *mus81* mutants due to incorrect segregation of chromosomes during meiosis I. The cytological defects of the *mus81* mutant during meiosis were largely corrected by the overexpression of RusA, a highly specific bacterial Holliday junction resolvase mentioned previously. RusA-D70N, which lacks endonuclease activity, but still binds Holliday junctions was unable to rescue the meiotic defects of fission yeast *mus81* mutants (Doe et al., 2000).

In vitro nuclease assays were carried out by incubation of the SpMus81-Eme1 with various substrates (Boddy et al., 2001). The results show that SpMus81-Eme1 is able to resolve Holliday junction into linear duplex products. The products reflect the essential feature of Holliday junction resolution, with the generation of nicks in the opposing strand that appears to be in correct polarity. The cleavage sites on each arm of the X-structure substrates have been mapped. All cleavage sites are within the homologous core or a few nucleotides 5' to that region, but never in the region of heterology 3' to the homologous core (Fig. 1-9). It suggests that SpMus81-Eme1 cuts X by introducing an incision on duplex DNA 5' to a double-strand/single strand junction. This mechanism is completely distinct from that of prokaryotic and mitochondrial resolvases, which have no requirement for a double-strand/single-strand junction (Lilley and White, 2001). All the genetic and biochemical evidence described above suggests that SpMus81 and Eme1 are essential components of a nuclear Holliday junction resolvase (Boddy et al., 2001).

Human Mus81 (hMus81) was isolated based on the significant evolutionary conservation of Mus81 (Chen et al., 2001). Human Mus81-associated endonuclease activity seemed to be dependent on the presence of a second subunit (Chen et al., 2001), and the second subunit was identified to be human Mms4 recently (Ogrunc and Sancar, 2003) . The same substrates as those in the SpMus81 assay were used to test the biochemical function of hMus81 (Chen et al., 2001). Like SpMus81-Eme1, hMus81 associated endonuclease activity fulfills the criterion set for a Holliday junction resolvase. It can cleave X-structures on strands of apparent polarity on each side of the junction, and most cleavage sites are symmetric near the junction. Despite resolution of X12 into linear products and the presence of a number of apparently symmetric cuts, the products cleaved by hMus81 are unable to be ligated. The same phenomena were also found with SpMus81-Eme1 cleavage products (Boddy et al., 2001). It indicates that the linear products have small gaps and flaps resulting from cuts that are close to the junction but they are not precisely symmetrical. Recent studies revealed two discrete Holliday junction resolvase activities in different fractions of Hela cell extracts, one corresponding to Mus81 and another corresponding to a resolvase which promotes branch migration/resolution reaction similar to RuvABC (Constantinou et al., 2002). In addition to their distinct chromatographic properties, the RuvABC-like activity was not depleted by anti-Mus81 polycolnal antibodies and the substrate specificities for the two activities were different (Constantinou et al., 2001).

These results are confusing. The major differences among the three Mus81 complexes are seen with their most relevant substrate, the Holliday junction. All three studies use essentially the same model Holliday junction, X12, which has a 12 bp branch-

migratable core (Fig. 1-9). This same junction structure has been used to study RuvC. The ScMus81-Mms4 complex cleaves the Holliday junction very poorly. However, SpMus81-Eme1 and human Mus81 cleave X12 well. The role of Mus81-Mms4/Eme1 in vivo is still unclear because of these controversial results.

1.5 Objectives of this study

Although early studies on the Mus81-Mms4/Eme1 complex provide valuable information for its characterization, the understanding of this complex is far from complete. Therefore, my research aims to make a contribution to our understanding of the Mus81-Mms4 complex in budding yeast. The objectives of this study are to characterize the structure and function of the budding yeast Mus81-Mms4 complex, and the specific aims of this study are:

(1) to identify whether the Mus81-Mms4 complex functions as a resolvase in budding yeast through epistatic analysis;

(2) to characterize the role of *MMS4* in mutagenesis;

(3) to map the domains required for interaction of Mus81 and Mms4;

(4) to determine the subcellular localization of Mus81 and Mms4 and the domains responsible for their localization.

```
ATGAGCCAGATCGTTGATTTTGTTGAGGACAAAGATTCAAGAAACGATGCCAGTATTCAGATCATCGATGGACCC   +75
 M  S  Q  I  V  D  F  V  E  D  K  D  S  R  N  D  A  S  I  Q  I  I  D  G  P     25
TCAAATGTTGAAATTATCGCTCTCTCGGAATCAATGGATCAAGATGAGTGCAAAAGGGCACATGTAAGCTCTGCA  +150
 S  N  V  E  I  I  A  L  S  E  S  M  D  Q  D  E  C  K  R  A  H  V  S  S  A     50
GAGATGATTCCATCATCACCGCAAAGAAAATCTGTTTCGAATGATGTTGAGAATGTTGACCTAAACAAATCTATC  +225
 E  M  I  P  S  S  P  Q  R  K  S  V  S  N  D  V  E  N  V  D  L  N  K  S  I     75
GAACTTTCTGCGCCGTTCTTCCAAGATATCAGTATAAGCAAGCTAGATGACTTTTCTACAACAGTAAATTCCATC  +300
 E  L  S  A  P  F  F  Q  D  I  S  I  S  K  L  D  D  F  S  T  T  V  N  S  I    100
ATAGATTCGTCTCTCAGAAATGAGAATAATGCAAAAGGAAACGCGAAAAAACTTTTGGATGATCTAATAAGTGAT  +375
 I  D  S  S  L  R  N  E  N  N  A  K  G  N  A  K  K  L  L  D  D  L  I  S  D    125
GAATGGTCAGCTGATCTTGAGTCGAGTGGAAAGAAGCACAACAAATCACAATACAATTTGAGGGACATCGCAGAG  +450
 E  W  S  A  D  L  E  S  S  G  K  K  H  N  K  S  Q  Y  N  L  R  D  I  A  E    150
AAATGGGGAGTACAGTCTTTAAAAAATCCAGAGCCTATTGCTGTTGACTGCGAATATAAGACACAAGGAATCGGA  +525
 K  W  G  V  Q  S  L  K  N  P  E  P  I  A  V  D  C  E  Y  K  T  Q  G  I  G    175
AAAACCAATAGTGACATTAGTGATAGTCCGAAATCACAGATAGGAGCAGCTGATATACTGTTCGATTTTCCACTG  +600
 K  T  N  S  D  I  S  D  S  P  K  S  Q  I  G  A  A  D  I  L  F  D  F  P  L    200
TCTCCAGTAAAACATGAGAACCCAACTGAAGAAAAGCACAATTCGATTGCTAATGAAAATTCTTCACCAGATAAT  +675
 S  P  V  K  H  E  N  P  T  E  E  K  H  N  S  I  A  N  E  N  S  S  P  D  N    225
AGCCTGAAACCAGCAGGAAAACAAAATCATGGTGAAGATGGGACATCCATGGCAAAAAGGGTATACAATAAAGGT  +750
 S  L  K  P  A  G  K  Q  N  H  G  E  D  G  T  S  M  A  K  R  V  Y  N  K  G    250
GAAGACGAGCAAGAACACCTTCCGAAAGGAAAGAAGAGAACCATAGCGTTATCAAGAACACTAATCAACAGCACC  +825
 E  D  E  Q  E  H  L  P  K  G  K  K  R  T  I  A  L  S  R  T  L  I  N  S  T    275
AAACTACCTGATACAGTAGAACTAAATCTTTCTAAATTTCTCGATTCCTCAGATAGTATTACTACTGATGTACTT  +900
 K  L  P  D  T  V  E  L  N  L  S  K  F  L  D  S  S  D  S  I  T  T  D  V  L    300
TCAACCCCTGCAAAGGGGTCTAACATAGTAAGGACAGGTAGTCAACCAATCTTTAGCAACGCTAATTGTTTTCAG  +975
 S  T  P  A  K  G  S  N  I  V  R  T  G  S  Q  P  I  F  S  N  A  N  C  F  Q    325
GAAGCAAAACGCTCGAAAACATTAACGGCTGAGGATCCCAAATGTACTAAAAATACTGCCAGAGAGGTATCACAA +1050
 E  A  K  R  S  K  T  L  T  A  E  D  P  K  C  T  K  N  T  A  R  E  V  S  Q    350
CTAGAGAATTATATTGCCTATGGGCAATACTATACTAGAGAAGACTCAAAAAACAAAATACGACACTTGTTAAAA +1125
 L  E  N  Y  I  A  Y  G  Q  Y  Y  T  R  E  D  S  K  N  K  I  R  H  L  L  K    375
GAAAACAAAAATGCTTTTAAGCGAGTTAACCAGATATATCGAGATAATATAAAAGCACGCTCTCAAATGATTATA +1200
 E  N  K  N  A  F  K  R  V  N  Q  I  Y  R  D  N  I  K  A  R  S  Q  M  I  I    400
GAGTTTTCGCCTAGCCTTCTCCAGTTATTTAAAAAAGGAGACAGTGATCTGCAACAACAATTGGCACCAGCAGTT +1275
 E  F  S  P  S  L  L  Q  L  F  K  K  G  D  S  D  L  Q  Q  Q  L  A  P  A  V    425
GTGCAATCAAGCTATAACGATTCTATGCCGCTTTTAAGATTTCTTCGAAAATGTGACAGTATTTACGACTTTAGT +1350
 V  Q  S  S  Y  N  D  S  M  P  L  L  R  F  L  R  K  C  D  S  I  Y  D  F  S    450
AACGATTTCTATTACCCCTGTGATCCCAAAATAGTTGAAGAAAACGTTTTGATTCTATATTATGATGCGCAAGAA +1425
 N  D  F  Y  Y  P  C  D  P  K  I  V  E  E  N  V  L  I  L  Y  Y  D  A  Q  E    475
TTTTTTGAACAATACACTTCACAAAAGAAAGAATTATATAGGAAGATACGATTTTTCTCAAAGAATGGAAAACAT +1500
 F  F  E  Q  Y  T  S  Q  K  K  E  L  Y  R  K  I  R  F  F  S  K  N  G  K  H    500
GTGATTCTTATACTAAGCGATATAAATAAACTCAAAAGAGCTATTTTCCAATTAGAAAATGAAAAGTACAAAGCT +1575
 V  I  L  I  L  S  D  I  N  K  L  K  R  A  I  F  Q  L  E  N  E  K  Y  K  A    525
AGGGTAGAACAACGATTGTCAGGAACAGAAGAAGCTTTAAGACCGAGAAGTAAAAAATCAAGCCAAGTTGGAAAA +1650
 R  V  E  Q  R  L  S  G  T  E  E  A  L  R  P  R  S  K  K  S  S  Q  V  G  K    550
TTAGGGATAAAAAAATTTGATTTAGAGCAACGATTGCGCTTCATTGATAGAGAATGGCATGTCAAAATACATACT +1725
 L  G  I  K  K  F  D  L  E  Q  R  L  R  F  I  D  R  E  W  H  V  K  I  H  T    575
GTAAATTCACATATGGAGTTTATTAATTCTCTGCCGAACCTAGTGTCATTAATTGGAAAACAGCGCATGGATCCC +1800
 V  N  S  H  M  E  F  I  N  S  L  P  N  L  V  S  L  I  G  K  Q  R  M  D  P    600
GCAATTCGGTATATGAAATATGCTCATTTGAATGTAAAATCCGCTCAGGATAGTACAGAAACGCTAAAGAAAACC +1875
 A  I  R  Y  M  K  Y  A  H  L  N  V  K  S  A  Q  D  S  T  E  T  L  K  K  T    625
TTTCATCAGATAGGGAGAATGCCTGAAATGAAGGCGAATAATGTCGTGAGCCTATACCCCAGTTTTCAATCATTA +1950
 F  H  Q  I  G  R  M  P  E  M  K  A  N  N  V  V  S  L  Y  P  S  F  Q  S  L    650
CTTGAAGATATTGAAAAGGGAAGACTGCAATCAGACAACGAAGGTAAATACTTGATGACTGAGGCAGTAGAAAAA +2025
 L  E  D  I  E  K  G  R  L  Q  S  D  N  E  G  K  Y  L  M  T  E  A  V  E  K    675
AGATTGTACAAACTGTTTACTTGTACTGATCCAAATGATACTATTGAATGATGATCGAACGAAACTTTGTATATA +2100
 R  L  Y  K  L  F  T  C  T  D  P  N  D  T  I  E  *                           691
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Figure 1-6. Nucleotide and deduced amino acid sequence of the *MMS4* gene. The nucleotide sequence of 2100 bp shown. The *MMS4* ORF is translated into a 691-amino acid protein with a calculated molecular weight of 78.7 kDa. Two consensus nuclear localization signals within the coding region, $KR(X)_{12}PKGKKR$ and $RPPSKK(X)_9$ KK, are underlined. The leucine repeat sequence is indicated by bold letters. Adopted from Xiao et al (1998).

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ATGGAACTCTCATCAAACTTAAAAGACCTATATATTGAATGGTTACAAGAATTAGTTGAC     +60
M  E  L  S  S  N  L  K  D  L  Y  I  E  W  L  Q  E  L  V  D       20
GGATTAACCCCTAAACAAGAACAACTCAAAATAGCCTATGAAAAAGCAAAAAGGAATTTA    +120
G  L  T  P  K  Q  E  Q  L  K  I  A  Y  E  K  A  K  R  N  L       40
CAAAATGCTGAAGGTTCATTTTATTATCCTACAGATCTAAAGAAAGTTAAGGGAATTGGC    +180
Q  N  A  E  G  S  F  Y  Y  P  T  D  L  K  K  V  K  G  I  G       60
AATACAATAATAAAGAGATTAGATACGAAATTACGGAACTATTGCAAAATTCACCACATA    +240
N  T  I  I  K  R  L  D  T  K  L  R  N  Y  C  K  I  H  H  I       80
TCTCCCGTTGAGGCTCCTTCCTTAACTCAGACAAGTAGCACCAGACCACCAAAGAGGACT    +300
S  P  V  E  A  P  S  L  T  Q  T  S  S  T  R  P  P  K  R  T      100
ACTACAGCTTTGCGTAGCATAGTAAACTCATGCGAAAATGATAAGAATGAAGCTCCCGAA    +360
T  T  A  L  R  S  I  V  N  S  C  E  N  D  K  N  E  A  P  E      120
GAGAAGGGAACTAAAAAGAGAAAAACTAGGAAGTATATACCCAAAAAAAGATCTGGAGGC    +420
E  K  G  T  K  K  R  K  T  R  K  Y  I  P  K  K  R  S  G  G      140
TACGCTATCCTTCTTTCCTTACTCGAGCTTAATGCCATTCCTCGAGGCGTTAGTAAAGAG    +480
Y  A  I  L  L  S  L  L  E  L  N  A  I  P  R  G  V  S  K  E      160
CAAATCATCGAGGTTGCAGGAAAATACAGTGACCATTGTATGACTCCAAATTTCTCCACA    +540
Q  I  I  E  V  A  G  K  Y  S  D  H  C  M  T  P  N  F  S  T      180
AAAGAATTCTACGGTGCTTGGTCGTCTATTGCTGCACTTAAAAAACATTCGTTGGTTCTT    +600
K  E  F  Y  G  A  W  S  S  I  A  A  L  K  K  H  S  L  V  L      200
GAGGAAGGTAGACCAAAACGGTATTCGCTAACAGAGGAAGGTGTAGAACTAACAAAGAGC    +660
E  E  G  R  P  K  R  Y  S  L  T  E  E  G  V  E  L  T  K  S      220
TTAAAGACGGCAGACGGAATCTCTTTTCCAAAAGAAAATGAAGAACCTAACGAGTATTCC    +720
L  K  T  A  D  G  I  S  F  P  K  E  N  E  E  P  N  E  Y  S      240
GTAACCAGGAATGAAAGTAGTGAATTCACAGCAAATCTGACTGACCTCCGTGGTGAATAT    +780
V  T  R  N  E  S  S  E  F  T  A  N  L  T  D  L  R  G  E  Y      260
GGTAAGGAAGAAGAGCCCTGCGATATAAATAACACTTCATTCATGCTGGATATAACTTTT    +840
G  K  E  E  E  P  C  D  I  N  N  T  S  F  M  L  D  I  T  F      280
CAAGATTTGAGCACGCCGCAAAGGCTGCAGAACAATGTATTTAAAAATGATAGACTGAAT    +900
Q  D  L  S  T  P  Q  R  L  Q  N  N  V  F  K  N  D  R  L  N      300
AGCCAAACTAATATATCTTCTCATAAATTGGAAGAGGTCTCTGATGATCAAACAGTACCT    +960
S  Q  T  N  I  S  S  H  K  L  E  E  V  S  D  D  Q  T  V  P      320
GATTCCGCATTAAAGGCTAAAAGCACAATAAAGAGGAGAAGGTACAACGGAGTAAGTTAC   +1020
D  S  A  L  K  A  K  S  T  I  K  R  R  R  Y  N  G  V  S  Y      340
GAATTGTGGTGTAGTGGTGATTTCGAAGTTTTCCCAATTATTGATCACAGAGAAATAAAG   +1080
E  L  W  C  S  G  D  F  E  V  F  P  I  I  D  H  R  E  I  K      360
TCACAATCTGATCGTGAGTTTTTTTCAAGGGCATTTGAAAGAAAAGGTATGAAGTCAGAG   +1140
S  Q  S  D  R  E  F  F  S  R  A  F  E  R  K  G  M  K  S  E      380
ATAAGGCAACTCGCTTTGGGTGATATTATATGGGTTGCCAAGAATAAAAATACCGGGTTG   +1200
I  R  Q  L  A  L  G  D  I  I  W  V  A  K  N  K  N  T  G  L      400
CAGTGTGTGCTCAACACCATAGTTGAAAGAAAAAGGCTAGACGATTTAGCTTTAAGTATA   +1260
Q  C  V  L  N  T  I  V  E  R  K  R  L  D  D  L  A  L  S  I      420
AGGGATAACAGGTTTATGGAGCAAAAAAATAGGTTAGAGAAATCTGGCTGTGAACACAAA   +1320
R  D  N  R  F  M  E  Q  K  N  R  L  E  K  S  G  C  E  H  K      440
TACTATCTCATTGAGGAGACTATGAGTGGCAACATTGGAAATATGAATGAGGCCCTAAAG   +1380
Y  Y  L  I  E  E  T  M  S  G  N  I  G  N  M  N  E  A  L  K      460
ACCGCGCTTTGGGTCATTTTAGTGTATTACAAATTCTCCATGATAAGAACTTGCAATTCG   +1440
T  A  L  W  V  I  L  V  Y  Y  K  F  S  M  I  R  T  C  N  S      480
GATGAAACTGTGGAAAAGATACATGCGTTGCATACTGTAATTTCTCATCACTATTCTCAA   +1500
D  E  T  V  E  K  I  H  A  L  H  T  V  I  S  H  H  Y  S  Q      500
AAAGATCTCATAGTTATATTTCCAAGTGACCTTAAAAGCAAGGACGATTATAAAAAGGTG   +1560
K  D  L  I  V  I  F  P  S  D  L  K  S  K  D  D  Y  K  K  V      520
CTTCTACAGTTTCGTCGAGAATTCGAACGAAAAGGCGGTATTGAATGCTGTCATAATCTT   +1620
L  L  Q  F  R  R  E  F  E  R  K  G  G  I  E  C  C  H  N  L      540
GAATGCTTTCAAGAACTAATGGGAAAAGGCGATCTAAAGACGGTTGGTGAGCTAACTATA   +1680
E  C  F  Q  E  L  M  G  K  G  D  L  K  T  V  G  E  L  T  I      560
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CATGTTTTAATGCTTGTTAAAGGTATATCTTTAGAAAAAGCAGTAGCCATCCAAGAAATA  +1740
H  V  L  M  L  V  K  G  I  S  L  E  K  A  V  A  I  Q  E  I      580
TTTCCTACCCTGAATAAAATACTAATGGCATATAAAACGTGCTCCTCGGAAGAAGAGGCT  +1800
F  P  T  L  N  K  I  L  M  A  Y  K  T  C  S  S  E  E  E  A      600
AAATTGTTGATGTTTAATGTACTGGGAGATGCACCTGGTGCGAAGAAAATTACTAAATCT  +1860
K  L  L  M  F  N  V  L  G  D  A  P  G  A  K  K  I  T  K  S      620
CTTTCAGAAAAGATATATGATGCTTTTGGTAAACTTTAG                       +1899
L  S  E  K  I  Y  D  A  F  G  K  L  *                           632
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Figure 1-7. Nucleotide and deduced amino acid sequence of the *MUS81* gene. The nucleotide sequence of 1899 bp is showen. The *MUS81* ORF is translated into a 632-amino acid protein with a calculated molecular weight of 72 kDa. Two Helix-hairpin-Helix motifs are underlined. The XPF endonuclease superfamily motif is indicated in bold letters.

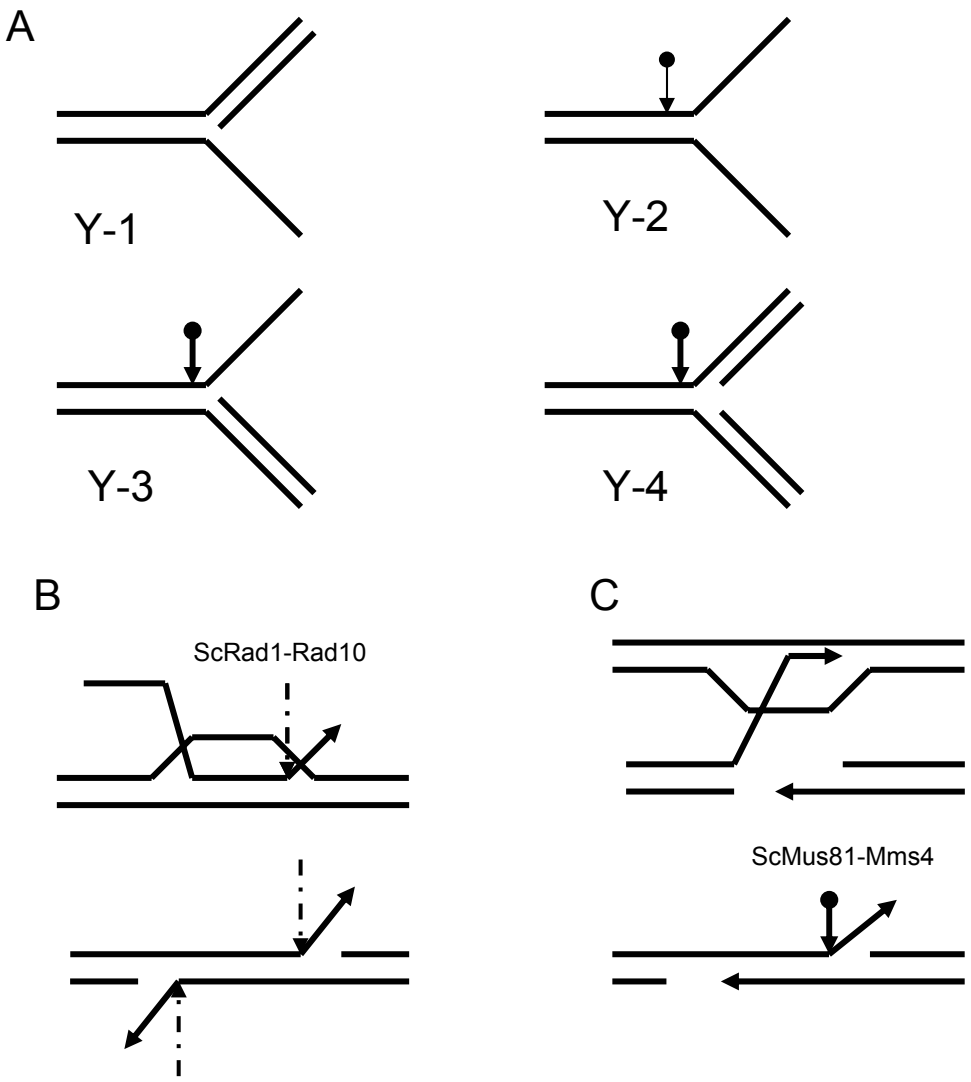


Figure 1-8. Cleavage of branched DNA molecules *in vitro* by ScMus81-Mms4 and ScRad1-Rad10. A. ScMus81-Mms4 shows much greater affinity for cleaving various Y junctions, with the relative intensity of cleavage indicated by the thickness of the arrow. B. ScRad1-Rad10 cleaves nonhomologous tails from the ends of ssDNA during strand invasion, and also removes such tails during SSA. C. ScMus81-Mms4 might also "clean up" intermediates of ssDNA annealing, where the newly synthesized DNA is longer than the gap into which it should anneal. Adopted from Haber (2001).

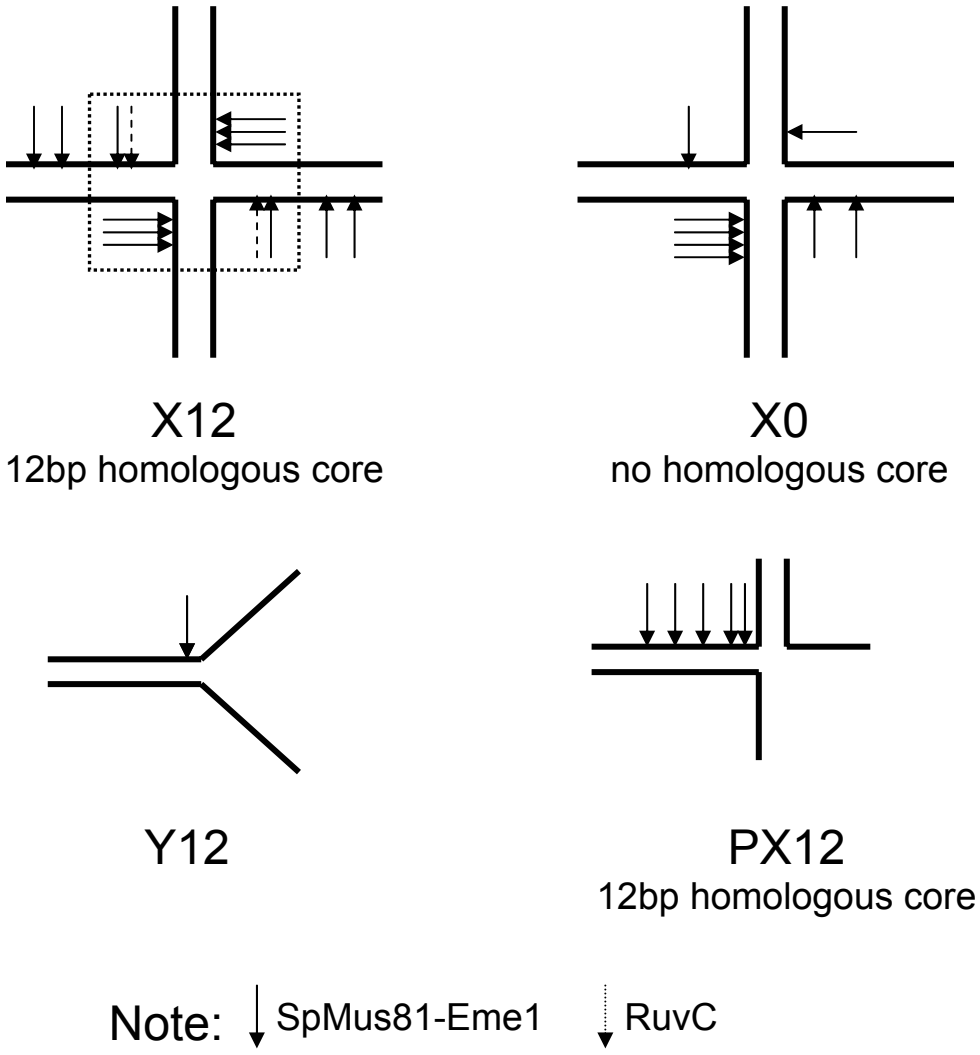


Figure 1-9. Cleavage of branched DNA molecules *in vitro* by SpMus81-Eme1 and RuvC. RuvC cleaves the X12 junction in a highly preferential way, producing ligatable ends. SpMus81-Eme1 and human Mus81 cleave predominantly, but not exclusively, within the region where each pair of arms has identical sequences (dashed line box) in which the crossover position can migrate. However, the cleavage products often have gaps or flaps so that the ends are usually unable to be ligated. Weaker cleavage is found in X0 with four unrelated arms. Adopted from Haber (2001).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Yeast Genetics

2.1.1 Yeast strains and cell culture

The *S. cerevisiae* strains used in this thesis are listed in Table 2-1. Yeast cells were cultured at 30°C either in a rich YPD medium or in a synthetic dextrose (SD) medium. YPD is a standard, complex medium composed of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. SD medium is used for selective growth of yeast auxotrophs. It contains 0.67% yeast nitrogen base without amino acids, 2% glucose, and addition of any necessary auxotrophic supplements. The necessary auxotrophic supplements includes 30 mg/L L-isoleucine, 150 mg/L L-valine, 20 mg/L adenine hemisulfate salt, 20 mg/L arginine HCl, 20 mg/L L-histidine HCl monohydrate, 100 mg/L L-leucine, 30 mg/L lysine HCl, 20 mg/L L-methionine, 50 mg/L L-phenylalanine, 200 mg/L L-threonine, 20 mg/L L-tryptophan, 30 mg/L L-tyrosine, 20 mg/L L-uracil. Any of the above auxotrophic supplements can be omitted to provide a selection media for yeast transformation. The auxotrophic supplements were made in 100 × stocks and added into media prior to autoclaving. To make plates, 2% agar was added to either YPD or SD medium prior to autoclaving.

Yeast cells can be stored for up to four months on plates sealed with parafilm at 4°C. For long term storage, yeast cells were grown in appropriate liquid medium (rich or

minimal media) at 30°C overnight. 0.7 ml of the culture was added into 0.3 ml of 50% sterile glycerol and then stored at -70°C.

2.1.2. Special media

Methyl methanesulfonate (MMS) was purchased form Aldrich (Milwaukee, USA), as an aqueous solution. To prepare MMS plates, MMS was added immediately before pouring the plates. In order to avoid problems caused by MMS degradation, MMS plates were usually freshly made and never stored for more than one day before plating.

2.1.3. Yeast transformation and targeted disruption

Yeast cells were transformed using a dimethyl sulfoxide (DMSO)-enhanced method as described (Hill et al., 1991). A 2 ml culture of yeast cells was grown overnight at 30°C in rich media (or appropriate minimal media), and subcultured into 3 ml of fresh media. When the yeast cells reached a mid-logarithmic phase of growth, they were pelleted by centrifugation. The yeast cells were washed in 400 µl LiOAc solution [0.1 M lithium acetate, 10 mM Tris-HCl (PH 8.0), 1 mM EDTA], and resuspended in 100 µl of the same solution. 5 µl of denatured carrier DNA (single stranded salmon sperm DNA) and 1-5 µl of transforming DNA were added and mixed well. After incubation at room temperature for 5 minutes, 280 µl of 50% of PEG4000 (50% polyethylene glycol 4000 in LiOAc solution) was added and mixed by inverting the tube 4-6 times. After the transformation mixture was incubated for 45 minutes at 30°C, 39 µl of DMSO was added, followed by a 5-minute heat shock in a 42°C waterbath. Yeast cells were then washed with sterile double distilled water (ddH_2O) and resuspended in 100 µl of ddH_2O. The

Table 2-1 *S. cerevisiae* strains

Strain	Genotype	Source
W303-10A	*MATa ade-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	H. Klein
W303-10D	*MATα ade-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*	H. Klein
WXY839	W303-10A with *mms4Δ::HIS3*	This study
WXY840	W303-10A with *mus81Δ::HIS3*	This study
LSY387	W303-10D with *rad52Δ::TRP1*	L. Symington
LSY395	W303-10D with *rad50Δ::hisG-URA3-hisG*	L. Symington
LSY401	W303-10D with *rad51Δ::LEU2*	L. Symington
LSY404	W303-10D with *rad54Δ::LEU2*	L. Symington
WXY841	W303 with *mms4Δ::HIS3 rad52Δ::TRP1*	This study
WXY842	W303 with *mms4Δ::HIS3 rad50Δ::hisG-URA3-hisG*	This study
WXY843	W303 with *mms4Δ::HIS3 rad51Δ::LEU2*	This study
WXY844	W303 with *mms4Δ::HIS3 rad54Δ::LEU2*	This study

Table 2-1 *S. cerevisiae* strains (continuted)

Strain	Genotype	Source
RKY2672	*MATa ura3-52 hisΔ200 trp1Δ63 leu2Δ1 ade8 lys2-Bgl hom3-10*	R.Kolodner
WXY845	RKY2672 with *mms4Δ::hisG-URA3-hisG*	This study
Y190	*MATa gal4 gal80 his3 trp1 ade2-101 ura3 leu2 ura3::GAL1-lacZ lys2::GAL1-HIS3*	D. Gietz

resuspended cells were plated on the appropriate minimal media. For targeted gene deletion, plasmid DNA was digested with restriction enzymes, precipitated by ethanol and resuspended in ddH_2O prior to transformation.

2.1.4 Sporulation and yeast tetrad dissection

In *rad52Δ*, *rad51Δ, rad54Δ* and *rad50Δ* mutants, homologous recombination is inactivated so that it would compromise targeted gene disruption. In order to disrupt the second gene in these mutants, genetic approaches were employed.

Two haploid strains with opposite mating types were streaked on YPD plates. Two days later, two strains were cross-streaked in an X-formation and mixed at the centre of the X on minimal selective plates which would support the growth of the diploid, but neither of the haploid cells. The plates were incubated at 30^{o}C for 2-3 days to obtain individual diploid colonies, which were inoculated into 2 ml of YPD medium and cells were allow to grow overnight at 30^{o}C with constant agitation and aeration. After incubation, cells were washed 2 times with sterile ddH_2O, resuspended in 5 ml sporulation media (0.5% potassium acetate, 0.5 × auxotrophic nutrients), and incubated at room temperature for 3 to 7 days with agitation and aeration.

Sporulation was checked by visual inspection with a light microscope after 3 days. If there were tetrads present, 10 µl of media was transferred to a sterile eppendorf tube and 10 µl of NEE-154 glusulase (Dupont Company, Wilmington, DE, USA) was added. After incubation at room temperature for 10 minutes, 20 µl of ice-cold ddH_2O was added and the tube was put on ice immediately. The tetrads were dissected on YPD plates by a Singer MSM micromanipulator (Singer Instrument Co. Somerset, England). The YPD

plates were incubated at 30°C for 3 days to allow the growth of tetrads. Each of the tetrads was replicated on different SD minimal plates to identify their genotypes.

2.1.5. Yeast genomic DNA isolation

To isolate genomic DNA for Southern hybridization, a protocol developed by Hoffman and Winston (Hoffman and Winston, 1987) was used. Yeast cells from liquid culture were collected by centrifugation and resuspended in 200 µl of extraction buffer [2% TritonX-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA, 0.01 M Tris HCl (pH8.0)]. One hundred µl of phenol, 100 µl of chloroform and 0.3 g of acid-washed glass beads were added to the cell mixture. The tube was then vortexed for 3 minutes (2 minutes for isolating plasmid DNA) at top speed. After a 5-minute centrifugation at top speed (13,200 rpm), the top aqueous layer was transferred to a clean eppendorf tube. To precipitate DNA, 2 volumes of 95% ethanol were added. The tube was stored at -20°C for 30 minutes and centrifuged for 15 minutes at top speed. The DNA pellet was dried and resuspended in 200 µl of ddH_2O, and then treated with 5 µl of RNase A (10 mg/ml stock) at 37°C for 10 minutes. The DNA was precipitated by adding 2 volumes of 95% ethanol and resuspended in 50 µl of ddH_2O.

2.1.6. Analysis of MMS sensitivity

A gradient plate assay is one of the methods to measure MMS sensitivity. 30 ml of molten YPD agar was mixed with an appropriate concentration of MMS to form the bottom layer. The gradient was created by pouring the media into tiled square petri dishes. After brief solidification for one hour, the petri dishes were returned flat and 30 ml of the same molten agar without MMS was poured to form the top layer. A 0.1 ml

sample was taken from an overnight culture, mixed with 400 µl sterile water and 0.5 ml of molten YPD agar, and then immediately imprinted onto freshly made gradient plates via a microscope slide. Gradient plates were incubated at 30°C for 2 days before photographs were taken.

MMS sensitivity was also measured by a serial dilution assay. Yeast cells were incubated in 5 ml of YPD medium overnight and a 500 µl aliquot was transferred to 5 ml of fresh medium. Cells were incubated at 30°C until they reached a mid-logarithmic phase. The cell density was adjusted to 2-5 × 10^6 cells/ml as determined by a hemocytometer, and further diluted serially by 10-fold with sterile ddH_2O. Five µl of each dilution was spotted onto YPD or YPD plates containing given concentrations of MMS. The plates were incubated at 30°C for 2 days and photographed.

2.1.7. DNA damage induced mutagenesis assays

Yeast strain RKY2672 contains two frameshift mutations, *hom3-10* with a 1-bp insertion, and *lys2-Bgl* with a 4-bp insertion. This strain contains a wild type *CAN1* suitable for the Can forward mutation analysis (Tishkoff et al., 1997). Single colonies of RKY2672 and its derivates were picked up from YPD medium, inoculated into a 5 ml YPD liquid culture and cells were grown overnight at 30°C with constant shaking. The cells were subcultured into 5 ml of fresh YPD and allowed to grow at 30°C until reaching mid-logarithmic phase. 0.05% MMS was added at that time and the tubes were put back to a 30°C shaker for a 15-minute incubation. The cells were washed once with 5% fresh sodium thiosulfate and twice with sterile water. Cells were then resuspended and serially diluted. One hundred µl cells from the appropriate dilution were plated on YPD, SD-Lys,

SD-Thr/Met and SD-Arg + canavanine plates. Colonies were counted after incubation at 30^{o}C for 3-5 days.

2.1.8. In vivo assay of protein interaction using yeast two-hybrid system

Yeast two-hybrid strain Y190 was transformed simultaneously with different combinations of pGBT-MUS81 constructs and pGAD-MMS4 constructs. The co-transformed colonies were streaked on SD-Leu/Trp/His plates to test the activation of *HIS3* gene. A filter assay was employed to determine the β-galactosidase (β-gal) activity (Bartel and Fields, 1995). For each combination, 5 independent co-transformants were resuspended in sterile ddH_2O at equal densities, spotted onto SD-Leu/Trp plates and allowed to grow for 3 days. Cells were transferred to a Whatman No.1 filter paper, immersed in liquid nitrogen for 10 seconds to permeablize cells, and placed on top of another filter which was presoaked with a mixture of 1.8 ml Z-buffer (16.1 g/L Na_2HPO_4. $7H_2O$, 5.50 g/L $NaH_2PO_4.H_2O$, 0.75 g/L KCl and 0.246 g/L MgSO4.$7H_2O$, pH 7.0) containing 5 µl β-mercaptoethanol and 45 µl of 20 mg/ml X-gal dissolved in *N,N*-dimethylformamide. Plates were sealed with parafilm and incubated at 30^{o}C. Color development was monitored during the 8 hours of incubation. Colony color development was scored as follows: ++++, less than 30 minutes; +++, 0.5-1 hour; ++, 1-2 hours; +, 2-4 hours. Colony color remaining unchanged after 8-hour incubation was considered negative and indicated as -.

2.1.9. Fluorescence studies

Exponential cultures of different transformed strains were grown in SD-Ura/Met liquid medium at 30°C. Cells were fixed by adding 1/10 volume of formaldehyde

directly to the medium (total formaldehyde concentration 3.7%; standard stock solution is 37%) and incubated cells at 30°C for at least another hour. Cells were pelleted and washed once with potassium phosphate (pH 7.5) and twice with PBS (pH 7.3). DNA was stained with 50 ng/ml DAPI (4' 6,-diamidino-2-phenylindole; Sigma) at room temperature for 10 minutes. After washing twice with PBS, 10 μl cells were spotted on slides. Cover slip was put on and sealed with clear nail polish. Pictures were captured on an Olympus fluorescence microscope equipped with a digital camera, and images were generated using Image-pro plus software (Mdeia Cybernetics).

2.2. Molecular Biology techniques

2.2.1 Bacterial culture and storage

The *E. coli* strain DH10B (GibcoBRL, Grand Island, NY, USA) was used for bacterial transformation. Since all of the plasmids used in this study contained the *bla* marker gene, transformed cells were cultured in LB liquid or agar media (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 1.2% agar for plates) containing 50 μg/ml of ampicillin (Amp). For short-term storage (2 or 3 months), transformed cells were stored on LB + Amp plates. For long-term storage, transformed cells were grown overnight in 900 μl of LB + Amp and immediately placed in a –70°C freezer after mixing with 100 μl of DMSO.

2.2.2. Preparation of competent cells

E. coli competent cells for electroporation were prepared as suggested in the BioRad *E. coli* Pulser manual. One liter of culture was incubated until an OD_{600nm} of 0.6 was

reached. The culture was centrifuged at 3500 rpm for 5 minutes in a Beckman GSA rotor and the pellet was resuspended in 500 ml of 10% ice-colded sterile glycerol. The centrifugation was repeated 4 times, with each pellet resuspended in a reduced volume; the last pellet was resuspended in 4 ml of ice-colded, sterile 10% glycerol. The cells were aliquoted into 1.5 ml eppendorf tubes to a volume of 25 µl, and were quickly placed in the –70^{o}C freezer for storage.

2.2.3. Bacterial transformation

All bacterial transformations in this study were carried out by the electroporation method. The DNA to be transformed was added to *E. coli* competent cells and the cell mixture was transferred to a pre-chilled electroporation cuvette (BioRad). After a brief incubation on ice, the cells were exposed to a voltage of 1.8 kV (for cuvettes with 0.1 mm width) using the *E. coli* Pulser (BioRad). 400 µl of SOC medium was added to the cuvette after electroporation. The cells were transferred to a 1.5 ml eppendorf tube, incubated at 37^{o}C for half an hour, spread on LB + Amp plates and incubated at 37^{o}C overnight.

2.2.4. Rapid preparation of plasmid DNA

Plasmid amplification and isolation was performed following the methods described by Maniatis et al. (1982). Single colonies were inoculated into 2 ml LB + Amp liquid media and grown overnight at 37^{o}C. Cells were collected by centrifugation and the pellet was resuspended in 350 µl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0). After mixing with 20 µl of lysozyme (10 mg/ml; Sigma,

St Louis MI), the mixture was quickly placed in a boiling water-bath for 40 seconds, followed by centrifugation for 10 minutes. The pellet was removed with a toothpick, and 8 µl of 5 M NaCl and 2 volumes of 95% ethanol were added to precipitate the DNA.

2.2.5. Agarose gel electrophoresis and DNA fragment isolation

For analysis of plasmid and genomic DNA, a 0.8% agarose gel was used in this study. Gels were run in 1 x TAE buffer (40 mM Tris-acetate, 2 mM Na_2EDTA) and stained in 0.5 µg/ml ethidium bromide (EtBr). DNA was visualized under UV light.

The method of DNA fragment isolation from agarose gels was adapted from Wang and Rossman (1994). After enzyme digestion, the sample was electrophoresed through 0.6% agarose gel and stained with EtBr. The band of interest was identified using an UV-illuminator and cut out of the gel. A 0.5 ml microcentrifuge tube was pierced at the bottom, and packed with chopped cheesecloth. The gel slice containing the DNA fragment was placed into the prepared tube, which was inserted into another 1.5 ml tube, left it in the $-70^{o}C$ freezer for 20 min and spun for 10 min at top speed. The flow through was extracted with an equal volume of phenol/chloroform (1:1) and then with chloroform. The DNA in the upper aqueous phase was precipitated by ethanol and resuspended in H_2O.

2.2.6 PCR amplification

Genomic DNA was isolated from yeast strain W303. The *MUS81* open reading frame was amplified using the primer MUS81-3 (5'-CCGGATCCATGGAACTCTCATCAAACTTAAAAG-3') and MUS81-4 (5'-

CCGTCGACTAAAGTTTACCAAAAGCATC-3'). The PCR reaction mixture consisted of 5 µl of 10 × PCR buffer, 5 µl of 2.5 mM dNTPs, 1 µl of MUS81-3 and MUS81-4 at 15 µM , 3 µl (about 100 ng) of genomic DNA, 2 µl 50 mM MgCl, 1 µl *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 32 µl sterile water. The PCR parameters were as follows: Step 1, 95°C for 5 minutes; Step 2, 95°C for 1 minute; Step 3, 45°C for 1 minute; Step 4, 72°C for 1.5 minute; Step 5, return to step 2 five times; Step 6, 95°C for 1 minute; Step 7, 52°C for 1 minute; Step 8, 72°C for 1 minute; Step 9, return to step 6 twenty-five times; Step 10, 72°C for 5 minutes.

2.2.7 DNA sequencing

Nucleotide sequences of the *MU81* open reading frame from PCR and other constructed plasmids were determined by the dideoxy chain termination method (Sanger et al., 1977) using a T7 DNA Polymerase Sequencing Kit (USB Corporation, Clereland, OH, USA).

2.2.8. Southern hybridization

After digestion of the genomic DNA with appropriate restriction enzymes, the DNA fragments were separated on a 0.8% agarose gel. The gel was then treated in a solution of 0.25 M HCl for 10 minutes for depurination, in 0.4 M NaOH/0.6 M NaCl for 30 minutes for denaturation, and in 1.5 M NaCl/0.5 M Tris-HCl (pH 7.5) for 30 minutes for neutralization. The DNA was transferred from the gel to a nylon-based membrane (GeneScreen Plus, DuPont) in the presence of 10x SSC (3M NaCl, 0.3 M tri-sodium citrate, pH 7.0) overnight.

The following day, the membrane was placed into a hybridization bottle with 5 ml of pre-hybridization solution [2x SSC, 10% dextran sulphate, 5x Denhardt's solution (50 × stock: 10 g $Ficoll_{400}$, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin; ddH_2O added to a total volume of 500 ml), 50% formamide, and 1% SDS] and incubated at 42°C in the hybridization oven for at least 2 hours. Before hybridization, 50 µl of boiled carrier DNA and 60 µl of probe were added to the prehybridization solution. The membrane was then incubated with the probe overnight at 42°C. The membrane was washed twice for 5 minutes at room temperature in 2x SSC/0.1% SDS and washed twice at 65°C in 0.2x SSC/ 0.1% SDS. The membrane was then exposed to X-ray film at -70°C with an intensifying screen.

The DNA probe was labelled with ^{32}P-dCTP using the Random Primer Labeling kit (GibcoBRL, Grand Island, NY, USA).

2.2.9. Plasmids construction

All plasmids used or constructed in this study are listed in Table 2-2. Plasmid manipulation was performed using enzymes from GibcoBRL and New England Biolabs as recommended by the manufacturers.

2.2.9.1 Plasmids for targeted gene deletion

Plasmid pGEM-MUS81 was constructed by cloning the *MUS81* ORF PCR product into the pGEM-T vector (Promega, Madison, WI, USA). Plasmid pGEM-MUS81 was digested by *Bgl*II to remove the fragment encoding aa51-501 and treated with Calf Intestinal Alkaline Phosphatase (CIP). The treated vectors were ligated with *HIS3* and

TRP1 markers respectively to form p*mus81Δ::HIS3* and p*mus81Δ::TRP1*. In all cases, nucleotides coding for amino acids 51-502 were deleted from the *MUS81* ORF.

To delete *MUS81* in host cells, both p*mus81Δ::HIS3* and p*mus81Δ::TRP1* were digested by *Hin*cII and *Ssp*I prior to yeast transformation.

To delete *MMS4*, p*mms4Δ::HIS3* (Xiao et al., 1998) was digested by *Eco*RI, and p*mms4::hisG-URA3-hisG* was digested by *Sal*I-*Pvu*II prior to yeast transformation.

2.2.9.2. <u>Plasmids for yeast two-hybrid assay</u>

In this system, two different sets of vectors were employed to assess protein-protein interactions. One set of vectors contain the Gal4 DNA-binding domain (BD), such as pGBT9 and its derivative pGBT9-Bg. *MUS81* and its truncated mutants were cloned in these vectors. Another set of vectors contain Gal4 DNA-activation domain (AD), namely pGAD424 and its derivative pGAD424-E. *MMS4* and relative mutants were cloned in these vectors.

A 1.9-Kb *Bam*HI –*Sal*I fragment from pGEM-MUS81 was cloned into the same sites of pGBT9-Bg to form pGBT-MUS81. To obtain the plasmid pGBT-MUS81 (290-632), the 1-Kb *Pst*I fragment from pGBT-MUS81 was cloned in the same site of pGBT9-Bg. The 1.1-Kb *Bgl*II fragment of pGBT-MUS81 was cloned in *Bgl*II digested pGBT-MUS81 (290-632) to give pGBT-MUS81 (138-632). Plasmid pGBT-MUS81 (1-288) was made by cleavage of pGBT-MUS81 with *Pst*I followed by self ligation. Plasmid pGBT-MUS81 was digested by *Eco*RI and slef-ligated to form pGBT-MUS81 (527-623).

The 0.6-Kb *Bgl*II fragment of pGBT-MUS81 (290-632) was cloned into the same site of pGBT9-Bg to form pGBT-MUS81 (290-502).

The 2.0-Kb *Pst*I fragment from pGBT-MMS4P was cloned into the *Pst*I site of pGAD424 to give pGAD-MMS4 (49-691). To make pGAD-MMS4 (1-336), the 1-Kb *Eco*RI-*Bam*HI fragment from pGBT-MMS4N was cloned into the same sites of pGAD424. The 2.0-Kb *Pst*I fragment from pGBT-MMS4P was cloned into the *Pst*I digested pGAD-MMS4 (1-366) to form pGAD-MMS4. The *EcoR*I-*Pst*I fragment from pGBT-MMS4N was cloned into the *EcoR*I-*Pst*I sites of pGAD424 to form pGAD-MMS4 (241-691). Plasmid pGAD-MMS4 was digested by *Bam*HI and self-ligated to form pGAD-MMS4 (336-598Δ). To obtain pGAD-MMS4 (598-691), the 2.0-Kb *Pst*I fragment from pGBT-MMS4P was cloned into the *Pst*I site of pGAD424-E, followed by *Bam*HI digestion and self-ligation. For constructing of pGAD-mms4-1, the 1-Kb *Eco*RI-*Bam*HI fragment from pGBT-mms4-1N was cloned into the same sites of pGAD424 to form a plasmid, and this plasmid was cleaved by *Bam*HI and ligated with the 0.78-Kb *Bam*HI fragment from pGAD-MMS4.

2.2.9.3 Plasmids for the localization of Mms4 and Mus81

Plasmid pUG36 (Niedenthal et al., 1996) was used as the vector to express Mms4 and Mus81 GFP fusion proteins in this study.

The 1.89-Kb *BamH*I-*Sal*I fragment from pGBT-MUS81 was cloned into the same sites of pUG36 to form pUG-MUS81. To obtain pUG-MUS81N (1-288) and pUG-MUS81C (288-631), the plasmid pUG36 was digested by *Sal*I and the cohensive ends were filled in by the Klenow fragment of *E. coli* DNA polymerase I, followed by the

digestion with *Bam*HI and used to clone the *Bam*HI-*Nae*I fragment from pGBT-MUS81 (1-288) and pGBT9-MUS81 (288-631) respectively.

The 2.4-Kb *Eco*RI-*Xho*I fragment from pGFP-MMS4 was cloned into the *Eco*RI and *Sal*I sites of pUG36 to form pUG-MMS4. To construct pUG-MMS4 (NLS1Δ), pGAD-MMS4 was digested by *Nco*I and the cohensive ends were filled in with Klenow, followed by digestion with *Hpa*I and self ligation to give pGAD-MMS4 (NLS1Δ); pUG36 was digested by *Sal*I and blunted with Klenow, followed by digested by *Eco*RI to form the treated pUG36 vector. Plasmid pGAD-MMS4 (NLS1Δ) was digested by *Bgl*II and the cohesive ends were filled in by Klenow, followed by digestion with *Eco*RI to release a 1.6Kb fragment. The fragment was cloned into the treated pUG36 vector to give pUG-MMS4 (NLS1Δ). To construct pUG-MMS4 (NLS2Δ), pGAD-MMS4 (336-598Δ) was digested by *Bgl*II and the cohesive ends were filled by Klenow, followed by digestion by *Eco*RI to release 1.3 Kb fragment, which was cloned into the treated pUG36 vector described above to form pUG-MMS4 (NLS2Δ).

2.2.10. Co-immunoprecipitation and Western blotting

Y190 cells transformed with two-hybrid constructs were grown in 10 ml SD-Trp/Leu liquid media at 30^{o}C overnight, subcultured in fresh SD-Trp/Leu media and allowed to grow at 30^{o}C until a cell density of OD_{600}=0.8 was reached. Yeast cells were harvested by centrifugation at 3000x g for 10 minutes at 4^{o}C. Yeast crude cell extract was made with YeastBuster protein extraction reagent (Novagen, Darmstadt, Germany) following the manufacturer's instruction.

The yeast crude cell extract was incubated with anti-Gal4-TA antibodies (Santa Cruz Biotechnology) at a ratio of 1:100 overnight at 4^{o}C. One hundred µl of PBS washed Protein-A beads (Sigma) were added and the tubes were gently agitated at 4^{o}C for at least 12 hours. After a brief centrifugation and removal of the supernatant, the protein-A beads were washed 5 times with ice cold PBS. The Protein-A beads were boiled, run on a 10% SDS PAGE gel, and then transferred to a PVDF mebrane (polyvinylidene difluoride, Bio-Rad Laboratories, Hercules, CA).

Membranes were treateded in a blocking solution (PBS, 3% non-fat milk) at room temperature for 1 hour. Anti- Gal4-DB antibodies (Santa Cruz Biotechnology) were diluted 1:1000 in 20 ml PBST [PBS, 0.05% Tween (v/v), 1% non-fat milk] and the PBST solution was incubated with membranes at 4^{o}C overnight. The second antibody, anti-mouse IgG conjugated with HRP, was used at a 1:5000 dilution. The Western Lightning Chemiluminescence Reagent (PerkimElmer Life Science, Boston, MA, USA) was utilized for detecting, and the membrane was then exposed to X-ray film.

Table 2-2 Plasmid constructs

Plasmid	Relevant characteristics	Source
pGEM-T	PCR T-vector	Promega
p*mus81Δ::HIS3*	*mus81Δ::HIS3* disruption cassette in pGEM	This study
p*mus81Δ::TRP1*	*mus81Δ::TRP1* disruption cassette in pGEM	This study
p*mms4Δ::HIS3*	*mms4Δ::HIS3* disruption cassette	W. Xaio
p*mms4Δ::HUH*	*mms4Δ::hisG-URA3-hisG* disruption cassette	W. Xiao
pGBT9	Yeast two-hybrid vector	Clontech
pGBT9-Bg	Yeast two-hybrid vector	W. Xiao
pGAD424	Yeast two-hybrid vector	Clontech
pGAD424-E	Yeast two-hybrid vector	W. Xiao
pGBT-MUS81	Full length *MUS81* in pGBT9-Bg	This study
pGBT-MUS81(290-632)	*mus81* (aa 290-632) in pGBT9-Bg	This study
pGBT-MUS81(138-632)	*mus81* (aa 138-632) in pGBT9-Bg	This study
pGBT-MUS81 (1-288)	*mus81* (aa 1-288) in pGBT9-Bg	This study

pGBT-MUS81(527-623)	*mus81* (aa 527-632) in pGBT9-Bg	This study
pGBT-MUS81 (290-502).	*mus81* (aa 290-502) in pGBT9-Bg	This study
pGBT9-MMS4P	*mms4* (aa 49-691) in pGBT9	W. Xiao
pGBT9-MMS4N	*mms4* (aa 1-336) in pGBT9	W. Xiao
pGAD-MMS4	Full length *MMS4* in pGAD424	This study
pGAD-MMS4 (49-691)	*mms4* (aa 49-691) in pGAD424	This study
pGAD-MMS4 (1-336)	*mms4* (aa 1-336) in pGAD424	This study
pGAD-MMS4 (241-691)	*mms4* (aa 241-691) in pGAD424	This study
pGAD-MMS4 (336-598Δ)	*mms4* (aa 336-598 deletion) in pGAD424	This study
pGAD-MMS4 (598-691)	*mms4* (aa 598-691) in pGAD424-E	This study
pGAD424-mms4-1	*mms4*-1 in pGAD424	This study
pUG36	GFP fusion protein expression vector	J. Hegemann
pUG-MUS81	Full length *MUS81* in pUG36	This study
pUG-MUS81N (1-288)	*mus81* (aa 1-288) in pUG36	This study
pUG-MUS81C (288-631)	*mus81* (aa 288-631) in pUG36	This study

pGFP-MMS4	Full length *MMS4* in pGFP	W. Xiao
pUG-MMS4	Full length *MMS4* in pUG36	This study
pUG-MMS4 (NLS1Δ).	*mms4* (aa 241-384 deletion) in pUG36	This study
pUG-MMS4 (NLS2Δ)	*mms4* (aa 336-598 deletion) in pUG36	This study

CHAPTER THREE

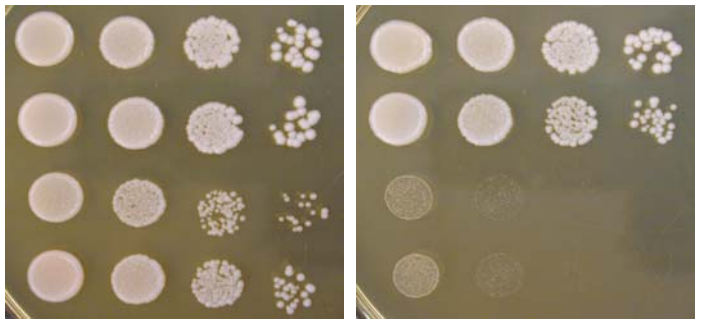
RESULTS

3.1. Epistatic analysis between *mms4* and mutations involved in recombination repair pathway

Biochemical studies on theMus81-Mms4 complex demonstrate that it functions as a branched molecule endonuclease. The branched structure is usually formed during recombination. Furthermore, previous studies in our laboratory suggest that *MMS4* does not belong to BER, NER and post-replication repair pathways (Xiao et al, unpublished data). Considering the above findings, it seems likely that Mus81-Mms4 is involved in the recombination repair pathway. Since Mus81 and Mms4 form a complex *in vivo* (Kaliraman et al., 2001; Mullen et al., 2001), and epistatic analysis indicates *MMS4* and *MUS81* function in the same DNA repair pathway (Mullen et al., 2001), *MMS4* was chosen to carry out the epistatic analysis with genes involved in the recombination repair pathway.

To determine the genetic relationship between *MMS4* and other genes involved in the recombination repair pathway, epistatic analyses were performed with *mms4* null mutation and other mutantions in the recombination repair pathway. The principle of epistatic analysis is that: if two genes belong to the same pathway, the double mutant defective in both genes will be no more sensitive to DNA damaging agents than one of the single mutants; otherwise, the two mutations will have additive or synergistic effects

A



W303 Wt

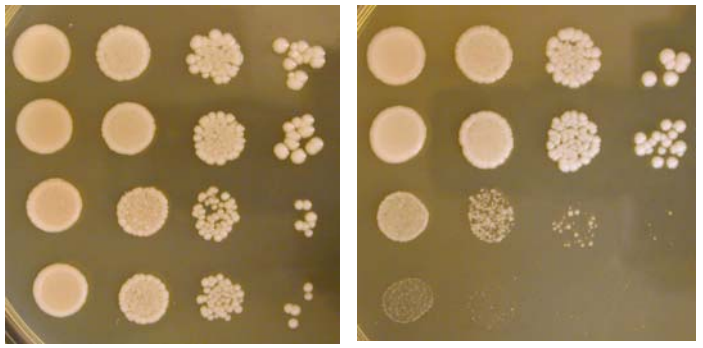
mms4Δ

rad52Δ

mms4Δ rad52Δ

YPD YPD+0.003% MMS

B



W303 Wt

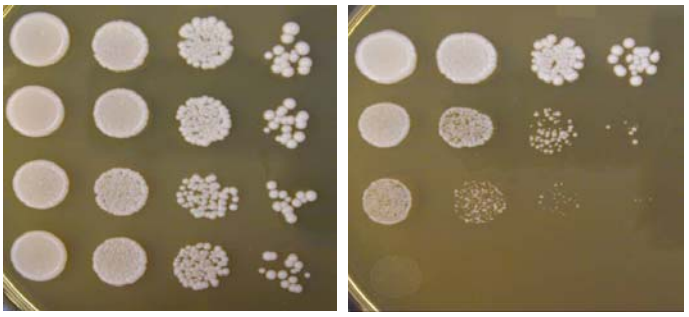
mms4Δ

rad50Δ

mms4Δ rad50Δ

YPD YPD+0.0025% MMS

C



W303 Wt

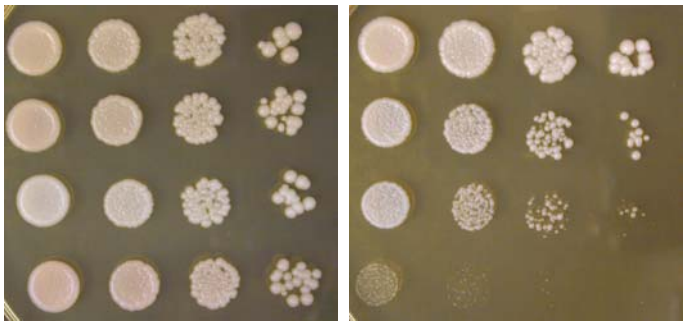
mms4Δ

rad51Δ

mms4Δ rad51Δ

YPD YPD+0.005% MMS

D



W303 Wt

mms4Δ

rad54Δ

mms4Δ rad54Δ

YPD YPD+0.005% MMS

Figure 3-1. Sensitivity of various mutants to MMS. Cells were cultured in YPD at 30^{o}C until they reached the log- phase. The 10-fold serial dilutions of the cell suspension were spotted onto YPD plates or YPD plates containing the indicated concentration of MMS. Incubation was carried out at 30^{o}C for 2 days before the plates were photographed. A, *rad52*; B, *rad50*; C, *rad51*; D, *rad54*

with respect to killing by DNA damaging agents. As shown in Fig. 3-1A, the *mms4Δ rad52Δ* double mutant displayed a similar level of sensitivity to MMS-induced killing as the *rad52Δ* single mutant, suggesting that *MMS4* belongs to the *RAD52* recombination repair pathway. In contrast, the *mms4Δ rad51Δ*, *mms4Δ rad54Δ* and *mms4Δ rad50Δ* double mutants were found to be more sensitive to MMS than either of the corresponding single mutants and the killing effects appeared to be additive (Fig 3-1 B, C, D), suggesting that MMS4 does not belong to any single pathways represented by *RAD50*, *RAD51* and *RAD54*.

3.2. The role of *MMS4* in mutagenesis

Since the Mus81-Mms4 complex plays an important role in the restart of DNA replication at stalled replication sites (Fabre et al., 2002; Haber and Heyer, 2001; Whitby et al., 2002), there are two possible means for the complex to resume the replication. One is an error-free pathway, whereas the other is an error-prone pathway. In the error–free pathway, replication resumed at the stalled replication forks and the original sequences are correctly restored. In the error-prone pathway, the stalled replication forks are resumed for the requirement of survival with the expense of an increased mutagenesis.

To understand the role of the budding yeast Mus81-Mms4 complex in mutagenesis, DNA damage induced mutagenesis assays were performed. These assays contained a forward mutation assay that detects mutations inactivating the arginine permease gene (Can^r mutations) and two reversion assays detecting mutations that either revert a 4-base insertion in the *LYS2* gene (*lys2-Bgl*) or revert a +1 T insertion in a stretch of 6 T's in the

Table 3-1. DNA damage induced mutation frequencies of *mms4* mutant

Strain	Survival	$Can^+/10^{-5}$	$Lys^+/10^{-7}$	$Hom^+/10^{-7}$
RKY2672 wild type	80.3%	1.3 ± 0.2	3.1 ± 0.4	2.1 ± 0.24
RKY2672 *mms4 Δ*	44.0%	1.1 ± 0.4	10.3 ± 2.5 (3.3)	7.4 ± 0.2 (3.5)

DNA damage induced mutagenesis assays are carried out as described in Materials and Methods. Numbers in parentheses refer to the fold increase in Lys^+ or Hom^+ revertants over the wild type control. The results represent an average of 5 independent experiments with standard deviation.

HOM3 gene (*hom3-10*) (Tishkoff et al., 1997). While the *mms4* mutation had little effect on the Can^r frequency, it increased the *lys2-Bgl* reversion by 3.3 fold and *hom3-10* reversion by 3.5 fold (Table 3-1). Furthermore, previous studies suggested that most of the Can^r mutations are base substitutions (Tishkoff et al., 1997). Thus the *MMS4* null mutation does not appear to alter MMS-induced base substitution, while it increases the frequency of frame-shift mutations. These results indicate that the Mus81-Mms4 complex may help to restart DNA replication at a stalled replication sites in a way which is able to prevent the frame-shift mutation. Taking into account that the Mus81-Mms4 complex is a branched molecule endonuclease and improper processing of the branched structure in the recombination repair pathway is likely to cause frame-shift mutations, these data also support the hypothesis that the Mus81-Mms4 complex helps to restart the stalled replication by cleavage of the 3' flap structure after strand displacement from the donor duplex (Fig. 1-8C) (Fabre et al., 2002).

3.3 Mapping interaction domains in Mms4 and Mus81

3.3.1 Interaction of Mms4 and Mus81 by a yeast two-hybrid assay

Gal4 contains a DNA-binding domain ($Gal4_{BD}$) within amino acids 1-147 and an activation domain ($Gal4_{AD}$) within amino acids 768-881. The coding regions for these two domains are carried on plasmids pGBT9 and pGAD424 respectively (Chien et al., 1991; Fields and Song, 1989). In order to test whether Mus81 and Mms4 form a complex *in vivo*, the *MUS81* and *MMS4* genes were tailored as in-frame fusions in each of the plasmids. The strain used for the two-hybrid assay carries a *GAL1-lacZ* fusion gene which contains an upstream UAS (upstream activation sequence) in the promoter region

of *GAL1*. The Gal4 DNA-binding domain is able to bind to the UAS. Interaction between a protein or peptide fused to $Gal4_{BD}$ and a second protein fused to the $Gal4_{AD}$ directs $Gal4_{AD}$ to the UAS site, resulting in β-galactosidase expression, which can be detected and measured by a β-gal filter assay.

For combinations of Mus81 or Mms4 fusions with appropriate paired control vectors, filter lifts remained pink (the color of yeast cells) during an 8-hour incubation at 30°C (Fig. 3-2). Filter lifts of transfomants carrying pGBT-MUS81 and pGAD-MMS4 turned blue within 30 minutes (Fig. 3-2). The results indicated a strong interaction between Mms4 and Mus81 *in vivo*.

3.3.2 The C-terminus of Mms4 is necessary and sufficient for its interaction with Mus81

In order to map the domain of Mms4 involved in the interaction with Mus81, a series of truncated $GAL4_{AD}$-Mms4 fusion constructs were made. These truncated constructs were tested for their ability to functionally complement the *mms4* mutation. The functional complementation analysis was performed by testing MMS induced killing of *mms4Δ* mutants transformed with various *MMS4* constructs on gradient plates containing 0.025% MMS (Fig. 3-3). If a truncated mutant remains functional, it will be able to complement the MMS sensitivity of the *mms4Δ* mutant. As shown in Fig. 3-3, the only deletion construct that complemented the MMS sensitivity of *mms4Δ* cells contains an *MMS4* coding region lacking the N-terminal 48 amino acids.

To carry out the yeast two-hybrid assay, these constructs were co-transformed with the full-length Mus81 ORF fused with a $Gal4_{BD}$ sequence. The β-gal filter assays were

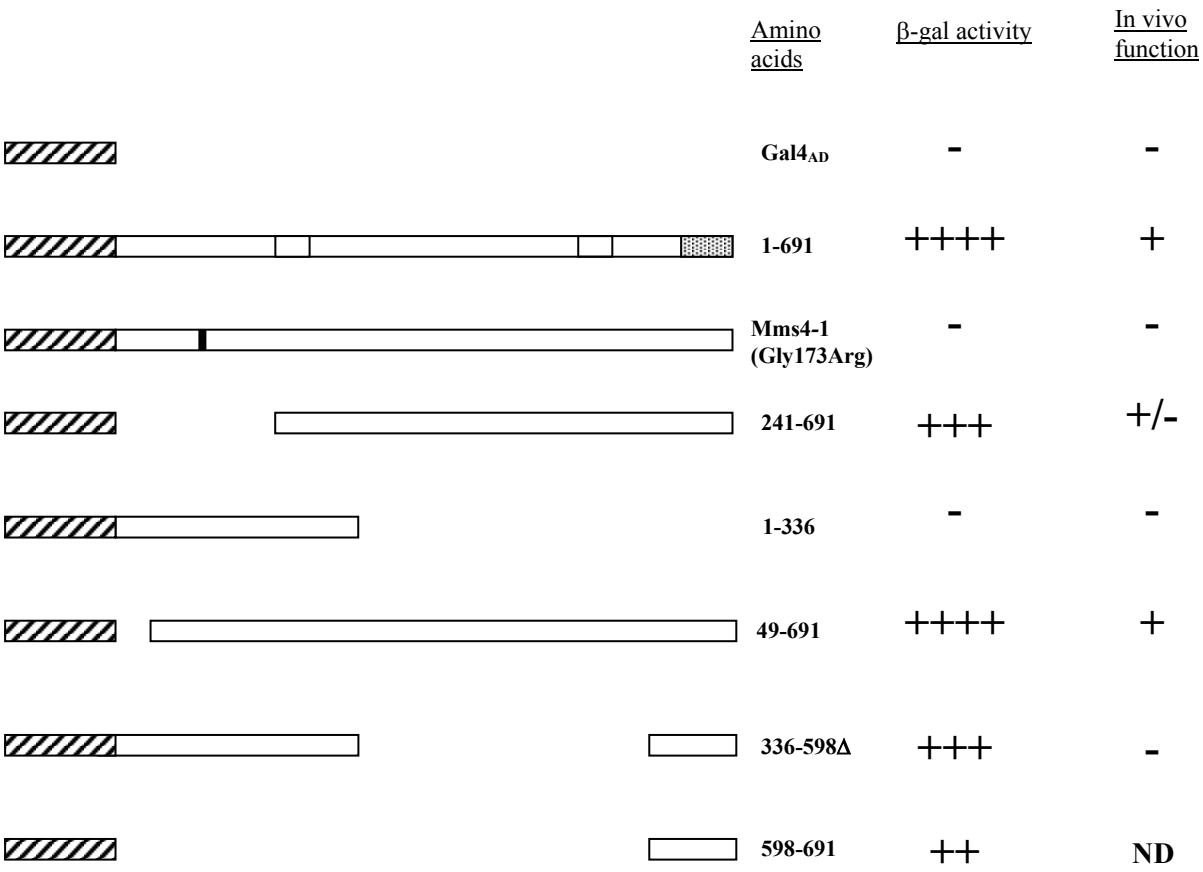

Amino acids
β-gal activity
In vivo function
Gal4AD
-
-
1-691
++++
+
Mms4-1 (Gly173Arg)
-
-
241-691
+++
+/-
1-336
-
-
49-691
++++
+
336-598Δ
+++
-
598-691
++
ND

Figure 3-2. Interactions of $Gal4_{BD}$-Mus81 and truncated $Gal4_{AD}$-Mms4 by a two-hybrid assay. Yeast strain Y190 was co-transformed with pGBT-MUS81 and various pGAD-MMS4 constructs, and the co-transformants were used for the β-gal assay. Y190 co-transformed with pGBT9 and pGAD-MMS4 was used as a negative control. The β-gal activity was determined by a colony filter assay and scored as described in Materials and Methods. Bars in each deletion constructs represent expected Mms4 fusion protein after deletion and numbers denote the amino acids remaining in the protein, or deletion (Δ) from the protein. Open boxes represent the putative nuclear localization signals, the dotted box represents the leucine repeat sequence, and the closed box indicates the location of Gly173Arg amino acid substitution.

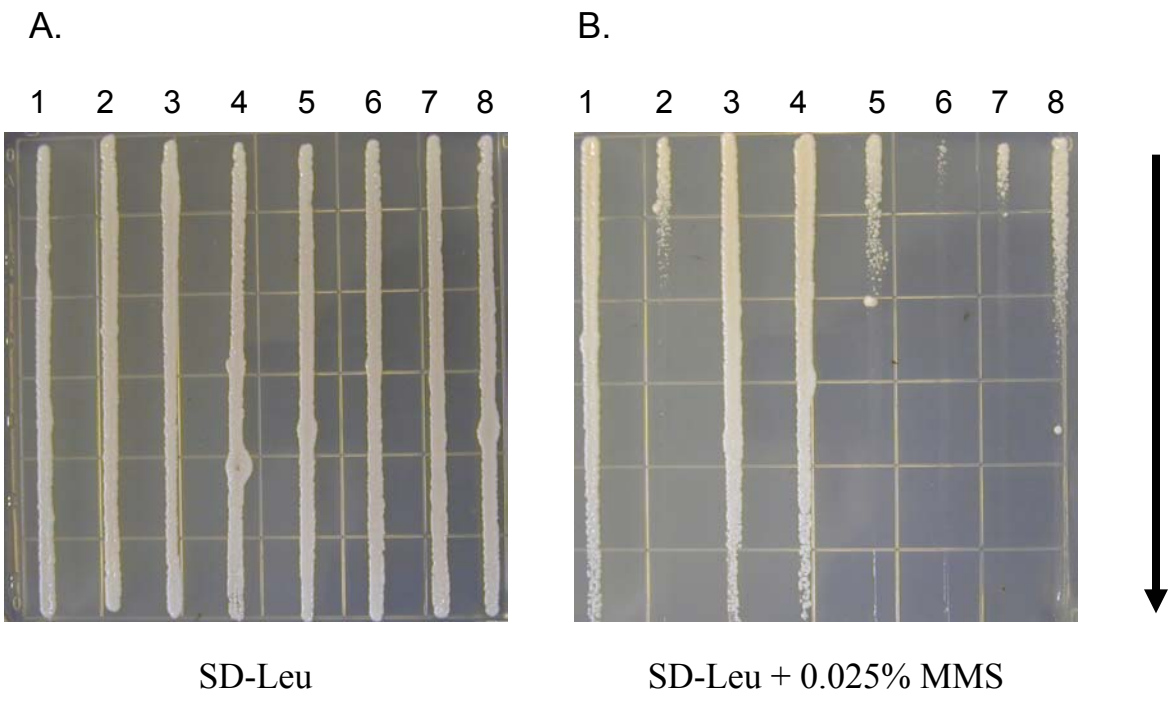


Figure 3-3. Functional complementation analysis of various truncated $GAL4_{AD}$- Mms4 fusion constructs and its deletion derivations. (A) SD-Leu control plate and (B) SD-Leu + 0.025% MMS gradient plate were incubated at 30°C for 2 days. Lane 1, W303 (wild type) + pGAD424; Lane 2, WXY839 (*mms4Δ*) + pGAD424; Lane 3, WXY839 + pGAD-MMS4; Lane 4, WXY839 + pGAD-MMS4 (49-691); Lane5, WXY839 + pGAD-MMS4-1; Lane 6, WXY839 + pGAD-MMS4 (336-598Δ); Lane 7, WXY839 + pGAD-MMS4 (1-336); Lane 8, WXY839 + pGAD-MMS4 (241-691). The arrow points towards the higher MMS concentration.

used to assess the interaction between the fusion proteins. As shown in Fig. 3-2, Mms4 is able to interact with Mus81. The Mms4 C-terminal deletion is unable to interact with Mus81, while truncated Mms4 proteins which retain the C-terminal, including all N-terminal and internal deletion mutants, are able to interact with Mus81, and the C-terminal 94 amino acids (residues 598-691) is sufficient for the interaction with Mus81. These results indicate that the C-terminus of Mms4 is important for its interaction with Mus81. However, the Gly173Arg mutant Mms4-1 is unable to interact with Mus81, although it has an intact C-terminus (Fig. 3-2). One possibility is that Mms4-1 is unstable in yeast cells and degraded very rapidly or does not produce full-length protein due to amino acid substitutions. The level of $Gal4_{AD}$-Mms4-1 was determined by Western analysis using anti-$Gal4_{AD}$ antibodies, and the inability to interact does not appear to be due to instability or truncation of Mms4-1. Since the Gly-to-Arg amino acid substitution is most likely to alter 3-D structure of the protein, it suggests that the 3-D structure of Mms4 is also important for the formation of the Mus81-Mms4 complex.

3.3.3 The C-terminus of Mus81 is sufficient for interaction with Mms4

As with Mms4, a series of GAL_{BD}-Mus81 deletion constructs were made to determine the domains of Mus81 involved in its interaction with Mms4. These truncated constructs were also tested for their ability to functionally complement the *mus81* mutation on 0.025% MMS gradient plates. As shown in Fig 3-4, no truncated mutants are able to complement the MMS sensitivity of the *mus81Δ* mutant. All the deletion constructs were paired with full length $Gal4_{AD}$-Mms4 for the two-hybrid analysis, and β-gal activity was determined by a filter assay. Results of the yeast two-hybrid assay show

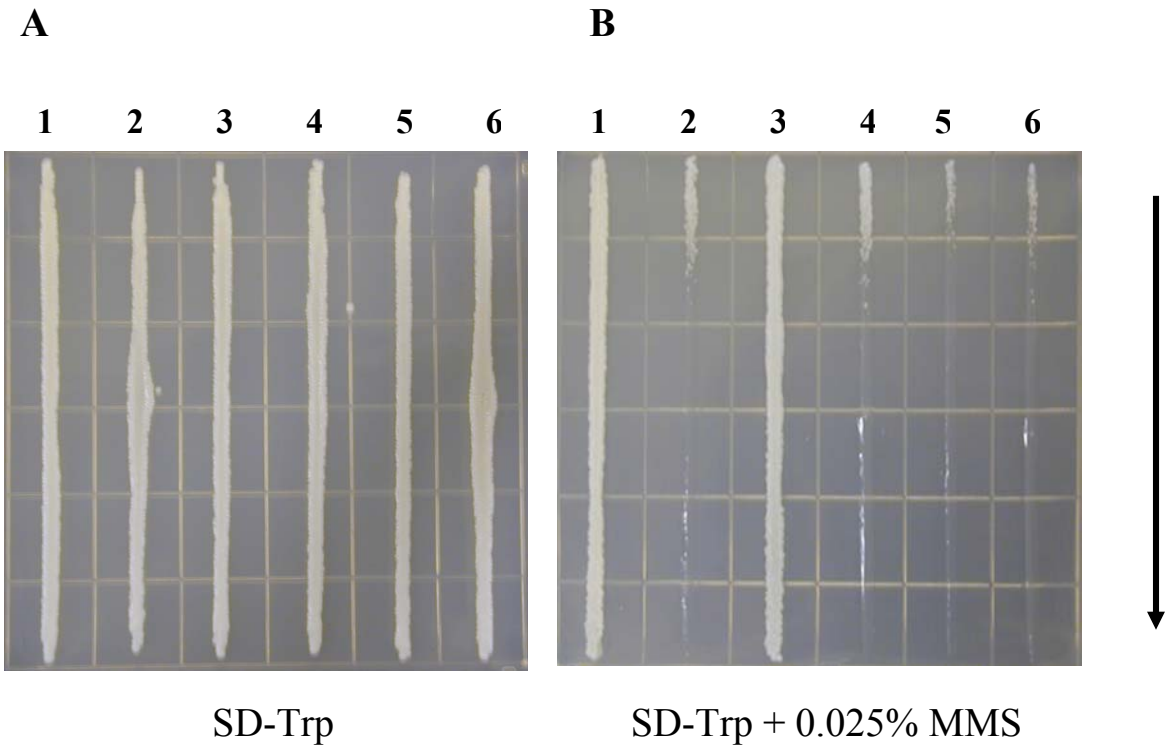


Figure 3-4 Functional complementation analysis of various truncated $Gal4_{BD}$- Mus81 fusion constructs. (A) SD-Trp control plate and (B) SD-Trp + 0.025% MMS gradient plate were incubated at 30^{o}C for 2 days. Lane 1, W303 (wild type) + pGBT9; Lane 2, WXY840 (*mus81Δ*) + pGBT9; Lane 3, WXY840 + pGBT-MUS81; Lane 4, WXY840 + pGBT-MUS81 (138-632); Lane5, WXY840 + pGBT-MUS81 (290-632); Lane 6, WXY840 + pGBT-MUS81 (1-290). The arrow points toward the higher MMS concentration.

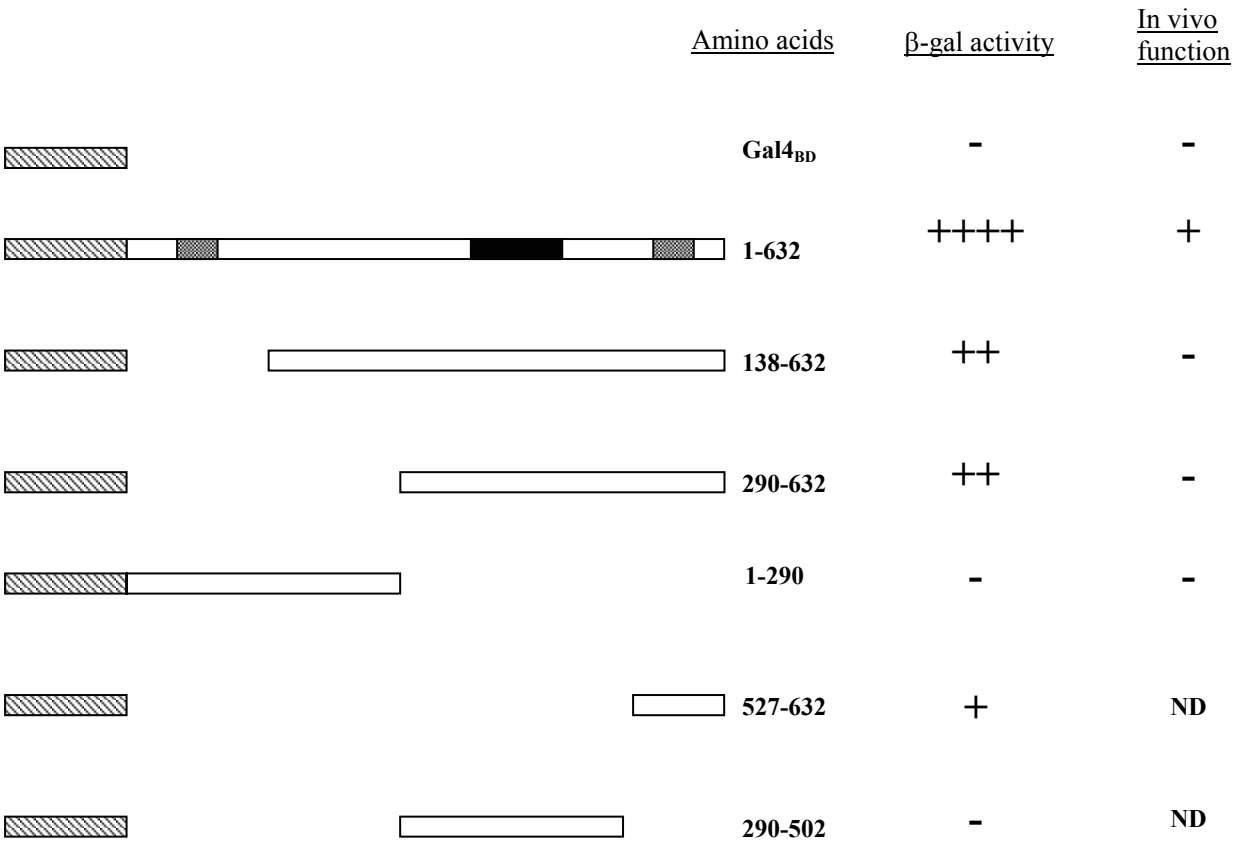


Figure 3-5. Interactions of $Gal4_{AD}$-Mms4 with various $Gal4_{BD}$- Mus81 constructs by a yeast two-hybrid assay. Yeast strain Y190 was co-transformed with pGAD-MMS4 and pGBT-MUS81 derivatives, and the co-transformants were used for the β-gal assay. Y190 co-transformed with pGBT-MUS81 and pGAD424 was used as a negative control. The β-gal activity was determined by a filter assay and scored as described in Materials and Methods. Bars in each deletion constructs represent expected Mus81 fusion protein after deletion and numbers denote the amino acids remained in the protein. Dotted boxes represent Helix-hairpin-Helix motifs and the closed box represents the XPF endonuclease domain.

that the C-terminally truncated mutant consisting of N-terminal 290 amino acids loses the interaction with Mms4 (Fig. 3-5). However, the N-terminally truncated mutants are able to interact with Mms4 and the C-terminal 106 amino acids (527-632) are sufficient for the interaction with Mms4. In contrast, the mutant consisting of 290-502 amino acids with an intact XPF endonuclease domain is unable to interact with Mms4, although the XPF domain is essential for the function of Mus81. The above results indicate that the C-terminal 106 amino acids of Mus81 contain the core interaction domain.

3.3.4 Mus81 is co-immunoprecipitated with Mms4

Results obtained from the yeast two-hybrid analysis mapped the regions involved in interaction between Mms4 and Mus81. To obtain additional supporting evidence, anti-$Gal4_{AD}$ antibodies were used in co-immunoprecipitation (Co-IP) experiments to pull down $Gal4_{BD}$-Mus81. In the control strain, no $Gal4_{BD}$-Mus81 is precipitated by $Gal4_{AD}$ alone, showing that most Mus81 is co-precipitated as a result of a specific interaction with Mms4. Full length Mms4 and all truncated mutants retaining the C-terminus were co-precipited with $Gal4_{BD}$-Mus81, while all C-terminally truncated mutants were not (Fig. 3-6). Conclusions drawn from Co-IP are consistent with those from yeast 2-hybrid assays.

3.4. Subcellular localization of Mms4 and Mus81

3.4.1 Both Mms4 and Mus81 are nuclear proteins

Since the Mus81-Mms4 complex is able to cleave and process DNA junctions at

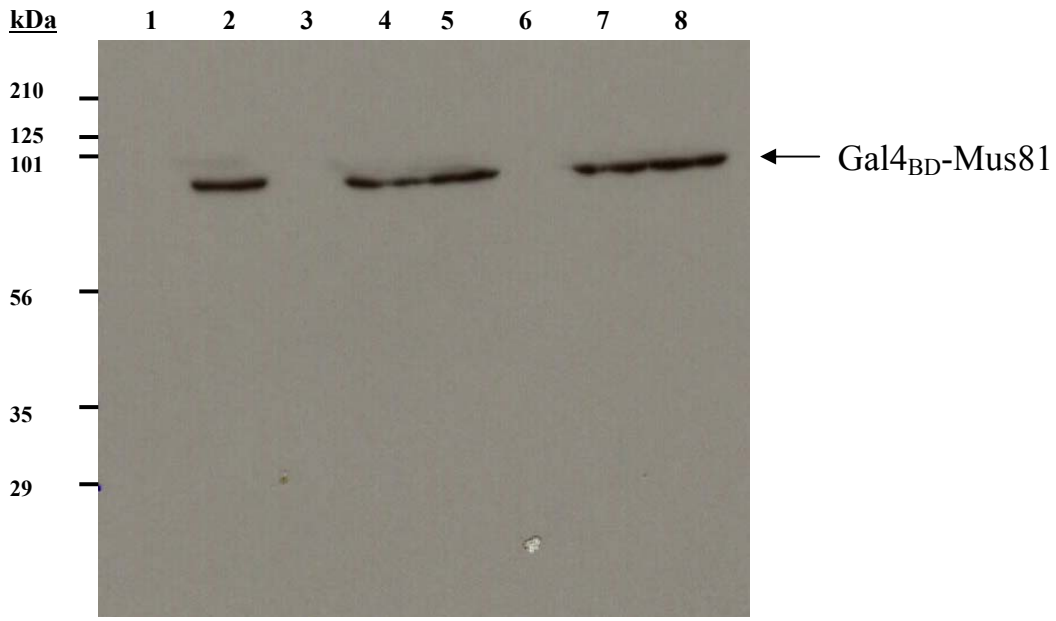


Figure 3-6 Immunoprecipitation of lysates from *S. cerevisiae* expressing $Gal4_{BD}$-Mus81 and truncated $Gal4_{AD}$-Mms4 proteins. The lysates were incubated with anti-$Gal4_{AD}$ antibodies, and then pulled down by Protein-A beads. Anti-$Gal4_{BD}$ antibodies were used for immunoblotting. The positions of molecular mass markers are indicated on the left.

Lane 1 pGBT-MUS81 + pGAD424;

Lane 2 pGBT-MUS81 + pGAD-MMS4;

Lane 3 pGBT-MUS81 + pGAD-MMS4-1;

Lane 4 pGAD-MUS81 + pGAD-MMS4 (49-691);

Lane 5 pGBT-MUS81 + pGAD-MMS4 (241-691);

Lane 6 pGBT-MUS81 + pGAD-MMS4 (1-336);

Lane 7 pGBT-MUS81 + pGAD-MMS4 (336-598Δ);

Lane 8 pGBT-MUS81 + pGAD-MMS4 (598-691).

areas of stalled replication, the two proteins are supposed to be localized in the nucleus. So far, their *in vivo* localization has not been demonstrated experimentally. In order to visualize the localization of Mms4 and Mus81 directly, the GFP-Mms4 and GFP-Mus81 fusion constructs were made. Fig. 3-7 shows that both Mms4 and Mus81 are indeed nuclear proteins.

3.4.2 NLS1 in Mms4 is necessary for its nuclear localization, but NLS2 is not required

There are two putative Nuclear localization sequence (NLS) within Mms4 (Xiao et al., 1998), NLS1, $KR(X)_{12}PKGKKR$ (amino acids 244-263), is very similar to those found in other yeast DNA repair proteins (Friedberg et al., 1995); NLS2 has the sequence $RPRSKK(X)_9KK$ (amino acids 539-555). In order to determine which putative bipartite nuclear transport sequences in Mms4 is responsible for the localization of Mms4, two truncated GFP-Mms4 fusion constructs were made, each with one of the two putative NLS deleted. Although the fragment (amino acids 336-598) containing NLS2 was deleted, the truncated protein was still able to localize in the nucleus (Fig. 3-8), suggesting that NLS2 is not necessary for the nuclear localization of Mms4. In contrast, when NLS1 was deleted, the truncated protein lost the ability to enter the nucleus and resulted in a uniform distribution throughout the cell (Fig. 3-9). These results suggest that NLS1 is necessary for the localization of Mms4, whereas NLS2 is not required.

3.4.3 The nuclear localization of Mus81 and Mms4 is independent of their interaction

As mentioned before, there are two putative nuclear localization sequences in

Mms4 (Xiao et al., 1998). From the amino acid sequence of Mus81, no classical NLS has been found, although the N-terminus is Lys/Arg rich. So it is possible that Mms4 forms a complex with Mus81 and then brings Mus81 into the nucleus. This hypothesis was tested by taking advantage of yeast cells with deletion of *MMS4*. When expressing the GFP-Mus81 fusion proteins in *mms4* null mutant strains, the fusion proteins were still localized in the nucleus (Fig. 3-10). Since the *mms4Δ* mutant strains lack Mms4, it confirms that the localization of Mus81 is independent of its interaction with Mms4. Fig. 3-10 shows GFP-Mms4 fusion proteins are also localized in the nucleus in *mus81Δ* mutant strains. From these data, it is concluded that the localization of Mms4 and Mus81 is independent of their interaction.

3.4.4 The N-terminal region in Mus81 is necessary for its localization

From the amino acid sequence of Mus81, no classical nuclear localization sequence (NLS) is found. There are several short stretches of basic residues (especially Lys or Arg) in the N-terminus of Mus81, which is known to help nuclear targeting of proteins (Christophe et al., 2000; Hicks and Raikhel, 1995; Macara, 2001). Thus it is likely that this region is responsible for the localization of Mus81. In order to delimit the region responsible for localization of Mus81, GFP-Mus81 truncations were made with N- and C-terminal deletions. The N-terminal half of GFP-Mus81 (amino acids 1-290) was still able to localize to the nucleus (Fig. 3-11), while the C-terminal half of GFP-Mus81 (amino acids 290-632) was unable to concentrate in the nucleus (Fig. 3-12). These results suggest that the N-terminus of Mus81 is required for its localization in the nucleus.

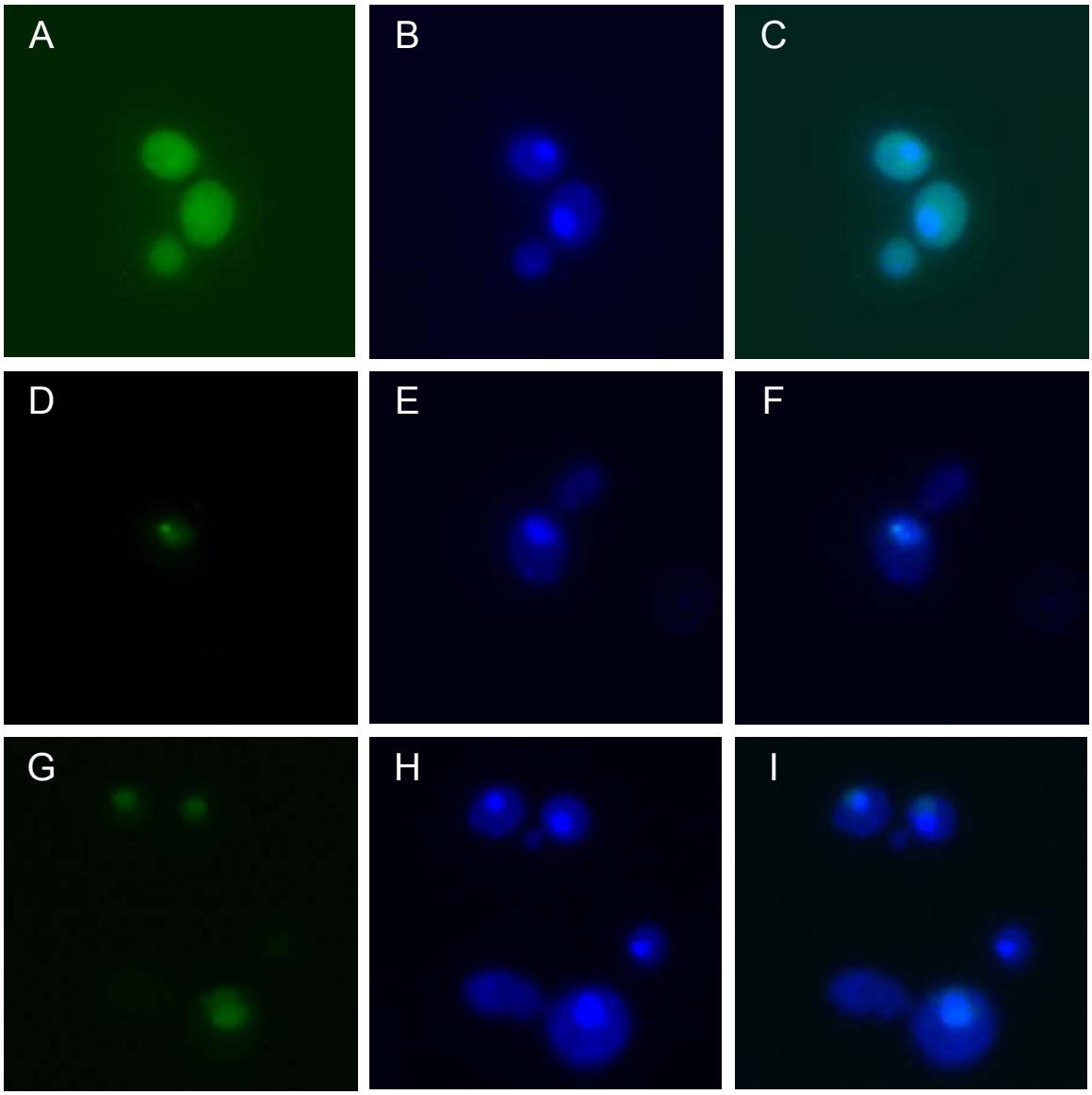


Figure 3-7. Both Mms4 and Mus81 are localized in the nucleus. A, B, C, yeast cells were transformed with pUG36 empty vector; D, E, F, yeast cells were transformed with pUG-MMS4; G, H, I, yeast cells were transformed with pUG-MUS81. GFP (A) or GFP-fusion proteins (B, C) and DNA stained by DAPI (B, E, H) were visualized by fluorescence microscopy. C is a merging of A and B; F is a merging of D and E; I is a merging of G and H.

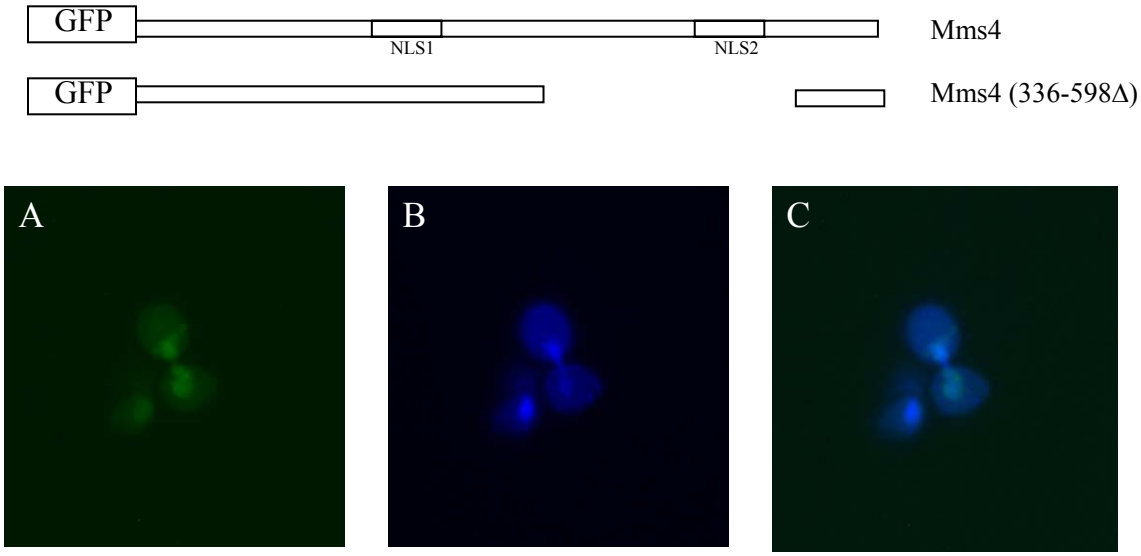


Fig. 3-8 The NLS2 is not required for the localization of Mms4. A, B, *mms4Δ* cells were transformed with pUG-MMS4 (336-598Δ). GFP-Mms4 fusion proteins (A) and DNA stained by DAPI (B) were visualized by fluorescence microscopy; C, The merged image of A and B.

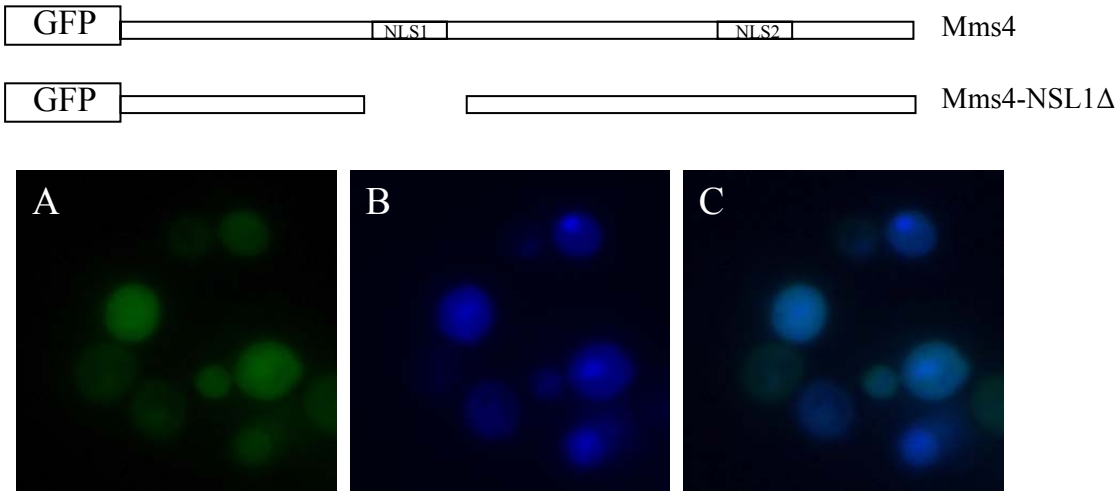


Figure 3-9. Deletion of NLS1 abolishes the localization of Mms4. A, B, *mms4Δ* cells were transformed with pUG-Mms4 (NLS1Δ). GFP-Mms4 (NLS1Δ) fusion proteins (A) and DNA stained by DAPI (B) were visualized by fluorescence microscopy; C, The merged image of A and B.

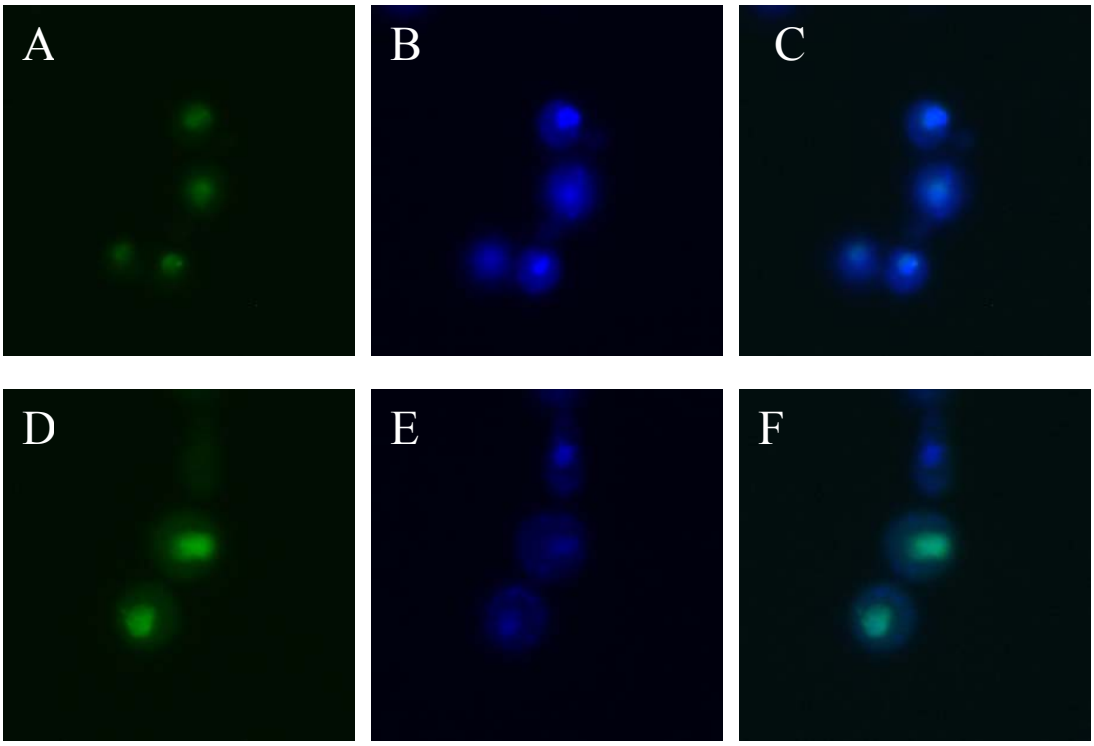


Figure 3-10. The localizations of Mms4 and Mus81 are independent of their interaction. A, B, *mms4Δ* cells were transformed with pUG-MUS81. GFP- Mus81 fusion proteins (A) and DNA stained by DAPI (B) were visualized by fluorescence microscopy; C The merged image of A and B. D, E, *mus81Δ* cells were transformed with pUG-MMS4. GFP-Mms4 fusion proteins (D) and DNA stained by DAPI (E) were visualized by fluorescence microscopy; F, the merged image of D and E.

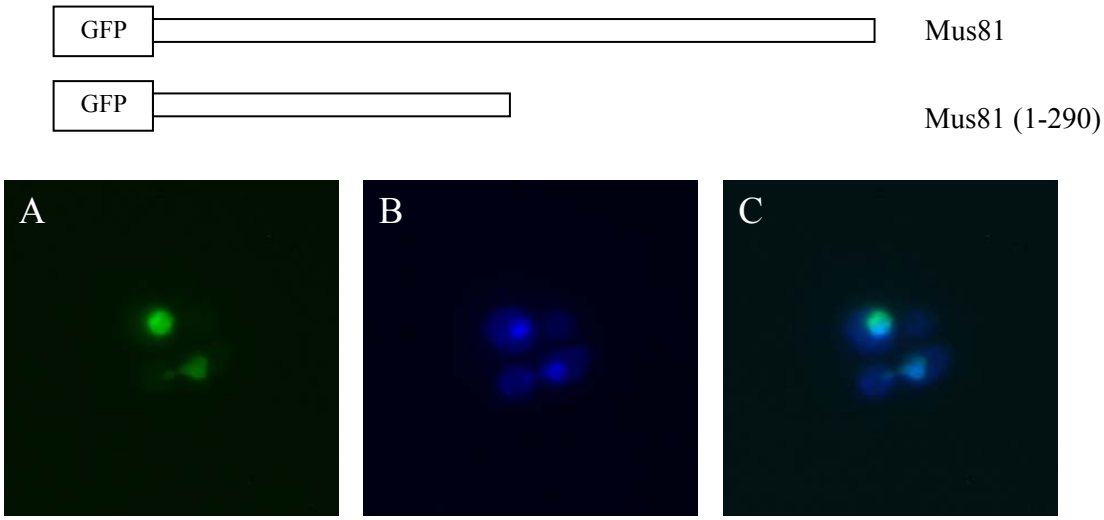


Figure 3-11. N-terminal of Mus81 is required for the localization. A, B, *mus81Δ* cells were transformed with pUG-MUS81 (1-290). GFP-Mus81 (1-290) fusion proteins (A) and DNA stained by DAPI (B) were visualized by fluorescence microscopy; C, The merged image of A and B.

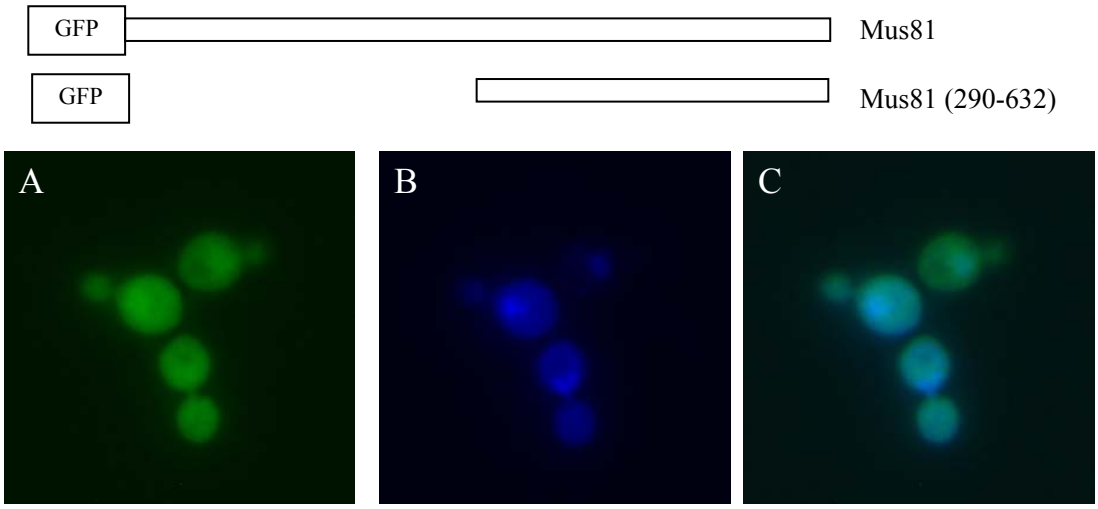


Fig 3-12. Deletion of the N-terminal of Mus81 abolishes its nuclear localization. A, B, *mus81Δ* cells were transformed with pUG-MUS81 (290-632). GFP-Mus81 (290-632) fusion proteins (A) and DNA stained by DAPI (B) were visualized by fluorescence microscopy; C, The merged image of A and B.

CHAPTER FOUR

DISCUSSION

4.1. Is the Mus81-Mms4 complex a *bona fide* Holliday junction resolvase?

It is generally accepted that the Mus81-Mms4 complex is a branched molecule endonuclease, but is it a *bona fide* Holliday junction resolvase? Genetic studies in *S. cerevisiae* are not fully compatible with this hypothesis. First, both *mms4Δ* and *mus81Δ* mutants are resistant to IR (Interthal and Heyer, 2000; Xiao et al., 1998). Although one could argue that IR-induced damage is repaired by mechanisms that do not require Holliday junction resolution, both *mms4* and *mus81* mutants also do not appear to display any defect in homologous recombination, since we obtained both deletion mutants by targeted disruption without apparent difficulty.

Second, we found that *rad52* is epistatic to *mms4* with respect to MMS killing, suggesting that *MMS4* belongs to the *RAD52* recombination repair pathway at least with respect to killing by MMS. It is known that the formation of a Holliday junction is an important step in the recombination DNA repair pathway. In budding yeast, a Holliday junction is initiated by the formation of a Rad51 nucleoprotein filament on single-stranded DNA (ssDNA). The Rad51 filament is active in homology searching and strand invasion in conjunction with Rad54. The 3' ends of the invading strands can then act as primers for the initiation of new DNA synthesis. This process leads to the formation of a Holliday junction. Thus, *RAD51* and *RAD54* are required for the formation of Holliday junction (Schwacha and Kleckner, 1997). When these genes are deleted, no Holliday

junction will be formed. If the Mus81-Mms4 complex functions as a resolvase *in vivo*, the *rad51 mms4* and *rad54 mms4* double mutants should show the same sensitivity as either of the corresponding single mutants. However, *mms4 rad54* and *mms4 rad51* double mutants show a marked increase in MMS sensitivity compared to corresponding single mutants. These results indicate that the Mus81-Mms4 complex may not function as a resolvase in budding yeast.

Besides the genetic studies mentioned above, some biochemical studies also suggest that the Mus81-Mms4 complex may not be a resolvase. *In vitro*, RuvC, RusA and the yeast mitochondrial enzymes Cce1 cleave Holliday junctions such as X12 in a precisely symmetrical pattern, often with strong sequence specificity (Lilley and White, 2001). Importantly, the cleaved ends can be religated. However, the Holliday junction endocleaved by Mus81-Mms4/Eme1 can not be ligated, indicating that the cleavage sites are not completely symmetrical (Constantinou et al., 2002), although cleavages of X12 appear to be at symmetrical sites (Boddy et al., 2001; Chen et al., 2001). Meanwhile, side-by-side comparison of Mus81-Eme1 and Mus81-Mms4 shows that these enzymes have the same substrate specificity, and the two enzymes cleave Holliday junctions relatively poorly compared to three- or four-way DNA junctions (Y-junctions) (Whitby et al., 2002). Fractionation of HeLa cell extracts revealed two discrete Holliday junction resolvase activities, one corresponding to Mus81 and another corresponding to the previously described resolvase that cofractionates with a branch migration activity, referred to as resolvase A (Constantinou et al., 2002; Constantinou et al., 2001). In addition to their distinct chromatographic properties, resolvase A activity was not depleted by anti-Mus81 polyclonal antibodies and the substrate specificities of the two

activities were different (Constantinou et al., 2002). Thus, the reason for the discrepancy between the *S. pombe* and *S. cerevisiae* data may be that budding yeasts could have another enzyme for resolving Holliday junctions and that this enzyme might be absent or less effective in *S. pombe*.

4.2. The interaction between Mms4 and Mus81

The results of refined mapping of the interaction domains of the Mms4 and Mus81 proteins indicate that these domains lie between amino acids 598-691 of Mms4 and 527-632 of Mus81. Screening the amino acid sequence of Mms4, a leucine repeat sequence, L-X_7-L-X_7-L-X_8-L-X_8-L, at the C-terminal region (Xiao et al. 1998) resemble a leucine/isoleucine repeat found at the C-terminus of Rad1 (Friedberg et al., 1995), which is probably required for the interaction of Rad1 with the Rad10 protein (Bardwell et al., 1993). Since Mms4 has limited amino acid sequence homology between its homologs in *S. pombe* and human, no such leucine repeat sequence is found in Eme1 and human Mms4 (hMms4). However, both Eme1 and hMms4 have a Leu/Ile rich C-terminus, indicating that their C-terminus may also be required for the interaction. Support for this hypothesis comes from studies by Boddy et al. (Boddy et al., 2001). In the yeast two-hybrid screen for the protein interacting with full length of SpMus81, one of the identified clones encoded the C-terminal half of Eme1.

Screening the amino acid sequence of Mus81, two helix-hairpin-helix motifs are found, one is found between the amino acid 527-632, another is found at the N-terminus. The one at the N-terminus has been demonstrated to be unnecessary for interaction with Mms4. The helix-hairpin-helix motif is found in many proteins involved in DNA

metabolism, and is known as a DNA binding motif (Doherty et al., 1996). Although this motif is possible to involved in protein-protein interaction (Nishino et al., 2003), the fact that the Mus81 DNA binding motif is contained in or adjacent to the Mms4 binding domain suggests that the binding of Mms4 to Mus81 may serve to precisely position Mus81 in contact with a specific DNA structure. This hypothesis is consistent with recent studies by Ogrunc and Sancar (2003) on human Mms4 and Mus81. Their results show that both hMus81 and hMms4 have no detectable nuclease activity, but the Mus81-Mms4 complex is a structure specific nuclease which is capable of resolving fork structures. Although no leucine repeat sequence is found at the interacting region of Mus81, this region is remarkably Leu/Ile rich, especially at the C-terminus (aa 558-632). Rad1 and Rad10 constitute a complex *in vivo* mediated by hydrophobic domains (Bardwell et al., 1993), and Leu and Ile are typical hydrophobic amino acids. Taken together, the Leu/Ile rich C-terminus of Mus81 seems to be responsible for the interaction with Mms4.

To our surprise, the Mms4-1 mutant protein loses its interaction with Mus81 *in vivo*, although it has an intact C-terminus as does Mms4. The Mms4-1 mutant protein contains a Gly173Arg substitution and loses all the functions of Mms4 (Xiao et al., 1998). Since this kind of amino acid substitution most likely causes the alteration in the 3-D structure of the protein, it suggests that the 3-D structure of Mms4 may be also important for the formation of a Mus81-Mms4 complex. From our results, the interaction between Mms4 and Mus81 seems to be mediated by hydrophobic domains is reminiscent of the interaction between Rad1 and Rad10 (Bardwell et al., 1993). If the 3-D structure of the protein is altered, it is likely to change the position of the hydrophobic region, thus

abolishing the interaction. Moreover, Mms4, Eme1 and hMms4 have limited amino acid sequence homology, and hMms4 was identified based on a comparison search of 3-D structures (Ogrunc and Sancar, 2003). It also suggests that the 3-D structure of Mms4 is a conserved feature important for its interaction with Mus81.

4.3. The subcelluar localization of Mms4 and Mus81

Both Mms4 and Mus81 are nuclear proteins. Because Mms4 contains two putative NLSs and no classical NLS is found in Mus81, we first hypothesized that Mms4 forms a complex with Mus81 in the cytoplasm, and then the complex translocates into the nucleus via the NLS of Mms4. Our observation modifies this hypothesis. The localization of Mms4 and Mus81 to the nucleus is independent of each other and does not rely on their interaction. Besides interacting with Mms4, Mus81 has been determined to interact with other proteins such as Cdc5, Rad53, Rad54, Cdc16 and Clb2 (Ho et al., 2002; Interthal and Heyer, 2000; Uetz et al., 2000). It remains possible that these proteins are able to assist Mus81 to enter the nucleus. From our results, the N-terminal half of Mus81 is able to mediate entry into the nucleus, and there are several short basic amino residue stretches in the N-terminal of Mus81. Thus, Mus81 is most likely has an ability to localize to the nucleus by itself. Proof of this hypothesis requires further experimentation.

4.4. Conclusions

From this study, several conclusions are drawn:

(1) *MMS4* belongs to the *RAD52* pathway, but does not belong to the single subpathway represented by *RAD51*, *RAD54* or *RAD50*, and Mus81-Mms4 seems not to be a resolvase *in vivo*.

(2) Mus81-Mms4 complex seems to resume the DNA replication at a stalled replication site in a way which can prevent the frameshift mutation.

(3) The C-terminus of Mms4 is required for the interaction with Mus81. The 3-D structure of Mms4 might be necessary for its interaction and essential functions.

(4) The C-terminus of Mus81 is required for the interaction with Mms4. The XPF endonuclease domain is not involved in the interaction.

(5) Both Mms4 and Mus81 localize in the nucleus. The putative NLS1 is responsible for the localization of Mms4. The interaction between Mms4 and Mus81 is not necessary for their nuclear localization. The N-terminus of Mus81 is required and sufficient for its nuclear localization.

REFERENCES

Aboussekhra, A., Chanet, R., Adjiri, A. and Fabre, F. (1992) Semidominant suppressors of Srs2 helicase mutations of Saccharomyces cerevisiae map in the RAD51 gene, whose sequence predicts a protein with similarities to procaryotic RecA proteins. *Mol Cell Biol*, **12**, 3224-3234.

Ajimura, M., Leem, S.H. and Ogawa, H. (1993) Identification of new genes required for meiotic recombination in Saccharomyces cerevisiae. *Genetics*, **133**, 51-66.

Alani, E., Padmore, R. and Kleckner, N. (1990) Analysis of wild-type and rad50 mutants of yeast suggests an intimate relationship between meiotic chromosome synapsis and recombination. *Cell*, **61**, 419-436.

Alani, E., Subbiah, S. and Kleckner, N. (1989) The yeast RAD50 gene encodes a predicted 153-kD protein containing a purine nucleotide-binding domain and two large heptad-repeat regions. *Genetics*, **122**, 47-57.

Alexiadis, V. and Kadonaga, J.T. (2002) Strand pairing by Rad54 and Rad51 is enhanced by chromatin. *Genes Dev*, **16**, 2767-2771.

Ames, B.N. and Shigenaga, M.K. (1992) Oxidants are a major contributor to aging. *Ann N Y Acad Sci*, **663**, 85-96.

Aravind, L., Walker, D.R. and Koonin, E.V. (1999) Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Res*, **27**, 1223-1242.

Balajee, A.S. and Bohr, V.A. (2000) Genomic heterogeneity of nucleotide excision repair. *Gene*, **250**, 15-30.

Bardwell, A.J., Bardwell, L., Johnson, D.K. and Friedberg, E.C. (1993) Yeast DNA recombination and repair proteins Rad1 and Rad10 constitute a complex in vivo mediated by localized hydrophobic domains. *Mol Microbiol*, **8**, 1177-1188.

Bardwell, A.J., Bardwell, L., Tomkinson, A.E. and Friedberg, E.C. (1994) Specific cleavage of model recombination and repair intermediates by the yeast Rad1-Rad10 DNA endonuclease. *Science*, **265**, 2082-2085.

Bartel, P.L. and Fields, S. (1995) Analyzing protein-protein interactions using two-hybrid system. *Methods Enzymol*, **254**, 241-263.

Bastin-Shanower, S.A., Fricke, W.M., Mullen, J.R. and Brill, S.J. (2003) The mechanism of mus81-mms4 cleavage site selection distinguishes it from the homologous endonuclease rad1-rad10. *Mol Cell Biol*, **23**, 3487-3496.

Baynton, K. and Fuchs, R.P. (2000) Lesions in DNA: hurdles for polymerases. *Trends Biochem Sci*, **25**, 74-79.

Bennett, R.J. and West, S.C. (1995) Structural analysis of the RuvC-Holliday junction complex reveals an unfolded junction. *J Mol Biol*, **252**, 213-226.

Boddy, M.N., Gaillard, P.H., McDonald, W.H., Shanahan, P., Yates, J.r. and Russell, P. (2001) Mus81-Eme1 are essential components of a Holliday junction resolvase. *Cell*, **107**, 537-548.

Boddy, M.N., Lopez-Girona, A., Shanahan, P., Interthal, H., Heyer, W.D. and Russell, P. (2000) Damage tolerance protein Mus81 associates with the FHA1 domain of checkpoint kinase Cds1. *Mol Cell Biol*, **20**, 8758-8766.

Bohr, V.A., Smith, C.A., Okumoto, D.S. and Hanawalt, P.C. (1985) DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. *Cell*, **40**, 359-369.

Bolt, E.L., Sharples, G.J. and Lloyd, R.G. (1999) Identification of three aspartic acid residues essential for catalysis by the RusA holliday junction resolvase. *J Mol Biol*, **286**, 403-415.

Broomfield, S., Hryciw, T. and Xiao, W. (2001) DNA postreplication repair and mutagenesis in Saccharomyces cerevisiae. *Mutat Res*, **486**, 167-184.

Buermeyer, A.B., Deschenes, S.M., Baker, S.M. and Liskay, R.M. (1999) Mammalian DNA mismatch repair. *Annu Rev Genet*, **33**, 533-564.

Chan, S.N., Vincent, S.D. and Lloyd, R.G. (1998) Recognition and manipulation of branched DNA by the RusA Holliday junction resolvase of Escherichia coli. *Nucleic Acids Res*, **26**, 1560-1566.

Chen, C. and Kolodner, R.D. (1999) Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants. *Nat Genet*, **23**, 81-85.

Chen, X.B., Melchionna, R., Denis, C.M., Gaillard, P.H., Blasina, A., Van de Weyer, I., Boddy, M.N., Russell, P., Vialard, J. and McGowan, C.H. (2001) Human Mus81-associated endonuclease cleaves Holliday junctions in vitro. *Mol Cell*, **8**, 1117-1127.

Chien, C.T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A*, **88**, 9578-9582.

Christophe, D., Christophe-Hobertus, C. and Pichon, B. (2000) Nuclear targeting of proteins: how many different signals? *Cell Signal*, **12**, 337-341.

Clever, B., Interthal, H., Schmuckli-Maurer, J., King, J., Sigrist, M. and Heyer, W.D. (1997) Recombinational repair in yeast: functional interactions between Rad51 and Rad54 proteins. *Embo J*, **16**, 2535-2544.

Connolly, B., Parsons, C.A., Benson, F.E., Dunderdale, H.J., Sharples, G.J., Lloyd, R.G. and West, S.C. (1991) Resolution of Holliday junctions in vitro requires the Escherichia coli ruvC gene product. *Proc Natl Acad Sci U S A*, **88**, 6063-6067.

Constantinou, A., Chen, X.B., McGowan, C.H. and West, S.C. (2002) Holliday junction resolution in human cells: two junction endonucleases with distinct substrate specificities. *EMBO J*, **21**, 5577-5585.

Constantinou, A., Davies, A.A. and West, S.C. (2001) Branch migration and Holliday junction resolution catalyzed by activities from mammalian cells. *Cell*, **104**, 259-268.

de Laat, W.L., Appeldoorn, E., Jaspers, N.G. and Hoeijmakers, J.H. (1998) DNA structural elements required for ERCC1-XPF endonuclease activity. *J Biol Chem*, **273**, 7835-7842.

de los Santos, T., Loidl, J., Larkin, B. and Hollingsworth, N.M. (2001) A role for MMS4 in the processing of recombination intermediates during meiosis in Saccharomyces cerevisiae. *Genetics*, **159**, 1511-1525.

Declais, A.C. and Lilley, D.M. (2000) Extensive central disruption of a four-way junction on binding CCE1 resolving enzyme. *J Mol Biol*, **296**, 421-433.

Doe, C.L., Dixon, J., Osman, F. and Whitby, M.C. (2000) Partial suppression of the fission yeast rqh1(-) phenotype by expression of a bacterial Holliday junction resolvase. *Embo J*, **19**, 2751-2762.

Doherty, A.J. and Jackson, S.P. (2001) DNA repair: how Ku makes ends meet. *Curr Biol*, **11**, 920-924.

Doherty, A.J., Serpell, L.C. and Ponting, C.P. (1996) The helix-hairpin-helix DNA-binding motif: a structural basis for non-sequence-specific recognition of DNA. *Nucleic Acids Res*, **24**, 2488-2497.

Dong, Z., Zhong, Q. and Chen, P.L. (1999) The Nijmegen breakage syndrome protein is essential for Mre11 phosphorylation upon DNA damage. *J Biol Chem*, **274**, 19513-19516.

Donovan, J.W., Milne, G.T. and Weaver, D.T. (1994) Homotypic and heterotypic protein associations control Rad51 function in double-strand break repair. *Genes Dev*, **8**, 2552-2562.

Duckett, D.R., Murchie, A.I., Bhattacharyya, A., Clegg, R.M., Diekmann, S., von Kitzing, E. and Lilley, D.M. (1993) The structure of DNA junctions and their interaction with enzymes. *Eur J Biochem*, **211**, 285-295.

Dunderdale, H.J., Benson, F.E., Parsons, C.A., Sharples, G.J., Lloyd, R.G. and West, S.C. (1991) Formation and resolution of recombination intermediates by E. coli RecA and RuvC proteins. *Nature*, **354**, 506-510.

Eadie, J.S., Conrad, M., Toorchen, D. and Topal, M.D. (1984) Mechanism of mutagenesis by O6-methylguanine. *Nature*, **308**, 201-203.

Eggleston, A.K. and West, S.C. (2000) Cleavage of holliday junctions by the Escherichia coli RuvABC complex. *J Biol Chem*, **275**, 26467-26476.

Eichman, B.F., Vargason, J.M., Mooers, B.H. and Ho, P.S. (2000) The Holliday junction in an inverted repeat DNA sequence: sequence effects on the structure of four-way junctions. *Proc Natl Acad Sci U S A*, **97**, 3971-3976.

Elborough, K.M. and West, S.C. (1990) Resolution of synthetic Holliday junctions in DNA by an endonuclease activity from calf thymus. *Embo J*, **9**, 2931-2936.

Emery, H.S., Schild, D., Kellogg, D.E. and Mortimer, R.K. (1991) Sequence of RAD54, a Saccharomyces cerevisiae gene involved in recombination and repair. *Gene*, **104**, 103-106.

Enzlin, J.H. and Scharer, O.D. (2002) The active site of the DNA repair endonuclease XPF-ERCC1 forms a highly conserved nuclease motif. *Embo J*, **21**, 2045-2053.

Esposito, M.S. (1978) Evidence that spontaneous mitotic recombination occurs at the two-strand stage. *Proc Natl Acad Sci U S A*, **75**, 4436-4440.

Fabre, F., Chan, A., Heyer, W.D. and Gangloff, S. (2002) Alternate pathways involving Sgs1/Top3, Mus81/ Mms4, and Srs2 prevent formation of toxic recombination intermediates from single-stranded gaps created by DNA replication. *Proc Natl Acad Sci U S A*, **99**, 16887-16892.

Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature*, **340**, 245-246.

Formosa, T. and Alberts, B.M. (1986) DNA synthesis dependent on genetic recombination: characterization of a reaction catalyzed by purified bacteriophage T4 proteins. *Cell*, **47**, 793-806.

Fortini, P., Parlanti, E., Sidorkina, O.M., Laval, J. and Dogliotti, E. (1999) The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. *J Biol Chem*, **274**, 15230-15236.

Frank, K.M., Sekiguchi, J.M., Seidl, K.J., Swat, W., Rathbun, G.A., Cheng, H.L., Davidson, L., Kangaloo, L. and Alt, F.W. (1998) Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. *Nature*, **396**, 173-177.

Friedberg, E.C., Walker, G. and Siede, W. (1995) *DNA repair and mutagenesis*. ASM Press, Washington, DC.

Furuse, M., Nagase, Y., Tsubouchi, H., Murakami-Murofushi, K., Shibata, T. and Ohta, K. (1998) Distinct roles of two separable in vitro activities of yeast Mre11 in mitotic and meiotic recombination. *Embo J*, **17**, 6412-6425.

Game, J.C. and Mortimer, R.K. (1974) A genetic study of x-ray sensitive mutants in yeast. *Mutat Res*, **24**, 281-292.

Giraud-Panis, M.J. and Lilley, D.M. (1997) Near-simultaneous DNA cleavage by the subunits of the junction-resolving enzyme T4 endonuclease VII. *Embo J*, **16**, 2528-2534.

Giraud-Panis, M.J. and Lilley, D.M. (1998) Structural recognition and distortion by the DNA junction-resolving enzyme RusA. *J Mol Biol*, **278**, 117-133.

Golin, J.E. and Falco, S.C. (1988) The behavior of insertions near a site of mitotic gene conversion in yeast. *Genetics*, **119**, 535-540.

Goodman, M.F. and Tippin, B. (2000) The expanding polymerase universe. *Nat Rev Mol Cell Biol*, **1**, 101-109.

Grawunder, U., Zimmer, D., Fugmann, S., Schwarz, K. and Lieber, M.R. (1998) DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. *Mol Cell*, **2**, 477-484.

Haber, J.E. and Heyer, W.D. (2001) The fuss about Mus81. *Cell*, **107**, 551-554.

Hargreaves, D., Rice, D.W., Sedelnikova, S.E., Artymiuk, P.J., Lloyd, R.G. and Rafferty, J.B. (1998) Crystal structure of E.coli RuvA with bound DNA Holliday junction at 6 A resolution. *Nat Struct Biol*, **5**, 441-446.

Hastings, P.J. (1988) Recombination in the eukaryotic nucleus. *Bioessays*, **9**, 61-64.

Hays, S.L., Firmenich, A.A. and Berg, P. (1995) Complex formation in yeast double-strand break repair: participation of Rad51, Rad52, Rad55, and Rad57 proteins. *Proc Natl Acad Sci U S A*, **92**, 6925-6929.

Hicks, G.R. and Raikhel, N.V. (1995) Protein import into the nucleus: an integrated view. *Annu Rev Cell Dev Biol*, **11**, 155-188.

Hill, J., Donald, K.A., Griffiths, D.E. and Donald, G. (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res*, **19**, 5791.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A.R., Sassi, H., Nielsen, P.A., Rasmussen, K.J., Andersen, J.R., Johansen, L.E., Hansen, L.H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sorensen, B.D., Matthiesen, J., Hendrickson, R.C., Gleeson, F., Pawson, T., Moran, M.F., Durocher, D., Mann, M., Hogue, C.W., Figeys, D. and Tyers, M. (2002) Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. *Nature*, **415**, 180-183.

Hoffman, C.S. and Winston, F. (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of Escherichia coli. *Gene*, **57**, 267-272.

Holliday, R. (1964) A mechanism for gene conversion in fungi. *Genet. Res.*, **5**, 282-304.

Hyde, H., Davies, A.A., Benson, F.E. and West, S.C. (1994) Resolution of recombination intermediates by a mammalian activity functionally analogous to Escherichia coli RuvC resolvase. *J Biol Chem*, **269**, 5202-5209.

Interthal, H. and Heyer, W.D. (2000) MUS81 encodes a novel helix-hairpin-helix protein involved in the response to UV- and methylation-induced DNA damage in Saccharomyces cerevisiae. *Mol Gen Genet*, **263**, 812-827.

Ivanov, E.L., Korolev, V.G. and Fabre, F. (1992) XRS2, a DNA repair gene of Saccharomyces cerevisiae, is needed for meiotic recombination. *Genetics*, **132**, 651-664.

Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A. and Shinagawa, H. (1991) Escherichia coli RuvC protein is an endonuclease that resolves the Holliday structure. *Embo J*, **10**, 4381-4389.

Jaskelioff, M., Van Komen, S., Krebs, J.E., Sung, P. and Peterson, C.L. (2003) Rad54p is a chromatin remodeling enzyme required for heteroduplex DNA joint formation with chromatin. *J Biol Chem*, **278**, 9212-9218.

Jiang, H., Xie, Y., Houston, P., Stemke-Hale, K., Mortensen, U.H., Rothstein, R. and Kodadek, T. (1996) Direct association between the yeast Rad51 and Rad54 recombination proteins. *J Biol Chem*, **271**, 33181-33186.

Johnson, R.D. and Symington, L.S. (1995) Functional differences and interactions among the putative RecA homologs Rad51, Rad55, and Rad57. *Mol Cell Biol*, **15**, 4843-4850.

Johzuka, K. and Ogawa, H. (1995) Interaction of Mre11 and Rad50: two proteins required for DNA repair and meiosis-specific double-strand break formation in Saccharomyces cerevisiae. *Genetics*, **139**, 1521-1532.

Jones, J.M., Gellert, M. and Yang, W. (2001) A Ku bridge over broken DNA. *Structure (Camb)*, **9**, 881-884.

Kaliraman, V., Mullen, J.R., Fricke, W.M., Bastin-Shanower, S.A. and Brill, S.J. (2001) Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease. *Genes Dev*, **15**, 2730-2740.

Kirkwood, T.B. (2002) Molecular gerontology. *J Inherit Metab Dis*, **25**, 189-196.

Kleff, S., Kemper, B. and Sternglanz, R. (1992) Identification and characterization of yeast mutants and the gene for a cruciform cutting endonuclease. *Embo J*, **11**, 699-704.

Klungland, A. and Lindahl, T. (1997) Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). *EMBO J*, **16**, 3341-3348.

Kodadek, T. and Wong, M.L. (1990) Homologous pairing in vitro initiated by DNA synthesis. *Biochem Biophys Res Commun*, **169**, 302-309.

Kogoma, T. (1996) Recombination by replication. *Cell*, **85**, 625-627.

Kogoma, T. (1997) Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. *Microbiol Mol Biol Rev*, **61**, 212-238.

Kolodner, R.D. and Marsischky, G.T. (1999) Eukaryotic DNA mismatch repair. *Curr Opin Genet Dev*, **9**, 89-96.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D. and Rehrauer, W.M. (1994) Biochemistry of homologous recombination in Escherichia coli. *Microbiol Rev*, **58**, 401-465.

Krejci, L., Damborsky, J., Thomsen, B., Duno, M. and Bendixen, C. (2001) Molecular dissection of interactions between Rad51 and members of the recombination-repair group. *Mol Cell Biol*, **21**, 966-976.

Krokan, H.E., Standal, R. and Slupphaug, G. (1997) DNA glycosylases in the base excision repair of DNA. *Biochem. J*, **325**, 1-16.

Kunkel, T.A., Meyer, R.R. and Loeb, L.A. (1979) Single-strand binding protein enhances fidelity of DNA synthesis in vitro. *Proc Natl Acad Sci U S A*, **76**, 6331-6335.

Kunkel, T.A., Silber, J.R. and Loeb, L.A. (1982) The mutagenic effect of deoxynucleotide substrate imbalances during DNA synthesis with mammalian DNA polymerases. *Mutat Res*, **94**, 413-419.

Le, S., Moore, J.K., Haber, J.E. and Greider, C.W. (1999) RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. *Genetics*, **152**, 143-152.

Lieber, M.R. (1997) The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *Bioessays*, **19**, 233-240.

Lilley, D.M. and White, M.F. (2001) The junction-resolving enzymes. *Nat Rev Mol Cell Biol*, **2**, 433-443.

Lin, F.L., Sperle, K. and Sternberg, N. (1984) Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. *Mol Cell Biol*, **4**, 1020-1034.

Lin, F.L., Sperle, K. and Sternberg, N. (1985) Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences. *Proc Natl Acad Sci U S A*, **82**, 1391-1395.

Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *Nature*, **362**, 709-715.

Lindahl, T., Sedgwick, B., Sekiguchi, M. and Nakabeppu, Y. (1988) Regulation and expression of the adaptive response to alkylating agents. *Annu Rev Biochem*, **57**, 133-157.

Lockshon, D., Zweifel, S.G., Freeman-Cook, L.L., Lorimer, H.E., Brewer, B.J. and Fangman, W.L. (1995) A role for recombination junctions in the segregation of mitochondrial DNA in yeast. *Cell*, **81**, 947-955.

Loeb, L.A. (1994) Microsatellite instability: marker of a mutator phenotype in cancer. *Cancer Res*, **54**, 5059-5063.

Loveless, A. (1969) Possible relevance of O-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. *Nature*, **223**, 206-207.

Lovett, S.T. (1994) Sequence of the RAD55 gene of Saccharomyces cerevisiae: similarity of RAD55 to prokaryotic RecA and other RecA-like proteins. *Gene*, **142**, 103-106.

Lovett, S.T. and Mortimer, R.K. (1987) Characterization of null mutants of the RAD55 gene of Saccharomyces cerevisiae: effects of temperature, osmotic strength and mating type. *Genetics*, **116**, 547-553.

Luo, G., Yao, M.S., Bender, C.F., Mills, M., Bladl, A.R., Bradley, A. and Petrini, J.H. (1999) Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A*, **96**, 7376-7381.

Macara, I.G. (2001) Transport into and out of the nucleus. *Microbiol Mol Biol Rev*, **65**, 570-594, table of contents.

Madhani, H.D., Bohr, V.A. and Hanawalt, P.C. (1986) Differential DNA repair in transcriptionally active and inactive proto-oncogenes: c-abl and c-mos. *cell*, **45**, 417-423.

Mahdi, A.A., Sharples, G.J., Mandal, T.N. and Lloyd, R.G. (1996) Holliday junction resolvases encoded by homologous rusA genes in Escherichia coli K-12 and phage 82. *J Mol Biol*, **257**, 561-573.

Mandal, T.N., Mahdi, A.A., Sharples, G.J. and Lloyd, R.G. (1993) Resolution of Holliday intermediates in recombination and DNA repair: indirect suppression of ruvA, ruvB, and ruvC mutations. *J Bacteriol*, **175**, 4325-4334.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular cloning: A laboratory manual. Cold Spring Habor Laboratory, Cold Spring Harbor, New York.

Marti, T.M., Kunz, C. and Fleck, O. (2002) DNA mismatch repair and mutation avoidance pathways. *J Cell Physiol*, **191**, 28-41.

Mazin, A.V., Alexeev, A.A. and Kowalczykowski, S.C. (2003) A novel function of Rad54 protein. Stabilization of the Rad51 nucleoprotein filament. *J Biol Chem*, **278**, 14029-14036.

Mazin, A.V., Zaitseva, E., Sung, P. and Kowalczykowski, S.C. (2000) Tailed duplex DNA is the preferred substrate for Rad51 protein-mediated homologous pairing. *Embo J*, **19**, 1148-1156.

McCullough, A.K., Dodson, M.L. and Lloyd, R. (1999) Initiation of base excision repair: glycosylase mechanisms and structures. *Annu. Rev. Biochem.*, **68**, 55-85.

McGill, C., Shafer, B. and Strathern, J. (1989) Coconversion of flanking sequences with homothallic switching. *Cell*, **57**, 459-467.

McGlynn, P. and Lloyd, R.G. (2000) Modulation of RNA polymerase by (p)ppGpp reveals a RecG-dependent mechanism for replication fork progression. *Cell*, **101**, 35-45.

Mellon, I. and Hanawalt, P.C. (1989) Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature*, **342**, 95-98.

Memisoglu, A. and Samson, L. (2000) Base excision repair in yeast and mammals. *Mutat Res*, **451**, 39-51.

Meselson, M.S. and Radding, C.M. (1975) A general model for genetic recombination. *Proc Natl Acad Sci U S A*, **72**, 358-361.

Milne, G.T. and Weaver, D.T. (1993) Dominant negative alleles of RAD52 reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes Dev*, **7**, 1755-1765.

Modrich, P. and Lahue, R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu Rev Biochem*, **65**, 101-133.

Moore, J.K. and Haber, J.E. (1996) Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. *Mol Cell Biol*, **16**, 2164-2173.

Moreau, S., Morgan, E.A. and Symington, L.S. (2001) Overlapping functions of the Saccharomyces cerevisiae Mre11, Exo1 and Rad27 nucleases in DNA metabolism. *Genetics*, **159**, 1423-1433.

Mortensen, U.H., Bendixen, C., Sunjevaric, I. and Rothstein, R. (1996) DNA strand annealing is promoted by the yeast Rad52 protein. *Proc Natl Acad Sci U S A*, **93**, 10729-10734.

Mullen, J.R., Kaliraman, V., Ibrahim, S.S. and Brill, S.J. (2001) Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in Saccharomyces cerevisiae. *Genetics*, **157**, 103-118.

Nairz, K. and Klein, F. (1997) mre11S--a yeast mutation that blocks double-strand-break processing and permits nonhomologous synapsis in meiosis. *Genes Dev*, **11**, 2272-2290.

Namsaraev, E. and Berg, P. (1997) Characterization of strand exchange activity of yeast Rad51 protein. *Mol Cell Biol*, **17**, 5359-5368.

Nasmyth, K.A. (1982) Molecular genetics of yeast mating type. *Annu Rev Genet*, **16**, 439-500.

Nelms, B.E., Maser, R.S., MacKay, J.F., ., Lagally , M.G. and Petrini, J.H. (1998) In situ visualization of DNA double-strand break repair in human fibroblasts. *Science*, **280**, 590-592.

Nelson, J.R., Lawrence, C.W. and Hinkle, D.C. (1996) Deoxycytidyl transferase activity of yeast REV1 protein. *Nature*, **382**, 729-731.

Niedenthal, R.K., Riles, L., Johnston, M. and Hegemann, J.H. (1996) Green fluorescent protein as a marker for gene expression and subcellular localization in budding yeast. *Yeast*, **12**, 773-786.

Nishino, T., Ariyoshi, M., Iwasaki, H., Shinagawa, H. and Morikawa, K. (1998) Functional analyses of the domain structure in the Holliday junction binding protein RuvA. *Structure*, **6**, 11-21.

Nishino, T., Komori, K., Ishino, Y. and Morikawa, K. (2003) X-Ray and Biochemical Anatomy of an Archaeal XPF/Rad1/Mus81 Family Nuclease. Similarity between Its Endonuclease Domain and Restriction Enzymes. *Structure*, **11**, 445-457.

Ogawa, T., Yu, X., Shinohara, A. and Egelman, E.H. (1993) Similarity of the yeast RAD51 filament to the bacterial RecA filament. *Science*, **259**, 1896-1899.

Ogrunc, M. and Sancar, A. (2003) Identification and characterization of human MUS81-MMS4 structure specific endonuclease. *J Biol Chem*.

Oram, M., Keeley, A. and Tsaneva, I. (1998) Holliday junction resolvase in Schizosaccharomyces pombe has identical endonuclease activity to the CCE1 homologue YDC2. *Nucleic Acids Res*, **26**, 594-601.

Orr-Weaver, T.L. and Szostak, J.W. (1983) Yeast recombination: the association between double-strand gap repair and crossing-over. *Proc Natl Acad Sci U S A*, **80**, 4417-4421.

Paques, F. and Haber, J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev*, **63**, 349-404.

Parsons, C.A., Stasiak, A., Bennett, R.J. and West, S.C. (1995) Structure of a multisubunit complex that promotes DNA branch migration. *Nature*, **374**, 375-378.

Paull, T.T. and Gellert, M. (1998) The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. *Mol Cell*, **1**, 969-979.

Paull, T.T. and Gellert, M. (1999) Nbs1 potentiates ATP-driven DNA unwinding and endonuclease cleavage by the Mre11/Rad50 complex. *Genes Dev*, **13**, 1276-1288.

Pellicer, A., Robins, D., Wold, B., Sweet, R., Jackson, J., Lowy, I., Roberts, J.M., Sim, G.K., Silverstein, S. and Axel, R. (1980) Altering genotype and phenotype by DNA-mediated gene transfer. *Science*, **209**, 1414-1422.

Perucho, M., Hanahan, D. and Wigler, M. (1980) Genetic and physical linkage of exogenous sequences in transformed cells. *Cell*, **22**, 309-317.

Petukhova, G., Stratton, S. and Sung, P. (1998) Catalysis of homologous DNA pairing by yeast Rad51 and Rad54 proteins. *Nature*, **393**, 91-94.

Petukhova, G., Sung, P. and Klein, H. (2000) Promotion of Rad51-dependent D-loop formation by yeast recombination factor Rdh54/Tid1. *Genes Dev*, **14**, 2206-2215.

Pfeiffer, P., Goedecke, W. and Obe, G. (2000) Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. *Mutagenesis*, **15**, 289-302.

Preston, B.D., Singer, B. and Loeb, L.A. (1986) Mutagenic potential of O4-methylthymine in vivo determined by an enzymatic approach to site-specific mutagenesis. *Proc Natl Acad Sci U S A*, **83**, 8501-8505.

Rafferty, J.B., Sedelnikova, S.E., Hargreaves, D., Artymiuk, P.J., Baker, P.J., Sharples, G.J., Mahdi, A.A., Lloyd, R.G. and Rice, D.W. (1996) Crystal structure of DNA recombination protein RuvA and a model for its binding to the Holliday junction. *Science*, **274**, 415-421.

Ranatunga, W., Jackson, D., Lloyd, J.A., Forget, A.L., Knight, K.L. and Borgstahl, G.E. (2001) Human RAD52 exhibits two modes of self-association. *J Biol Chem*, **276**, 15876-15880.

Raymond, W.E. and Kleckner, N. (1993) RAD50 protein of S.cerevisiae exhibits ATP-dependent DNA binding. *Nucleic Acids Res*, **21**, 3851-3856.

Resnick, M.A. and Martin, P. (1976) The repair of double-strand breaks in the nuclear DNA of Saccharomyces cerevisiae and its genetic control. *Mol Gen Genet*, **143**, 119-129.

Rodriguez, K., Wang, Z., Friedberg, E.C. and Tomkinson, A.E. (1996) Identification of functional domains within the RAD1.RAD10 repair and recombination endonuclease of Saccharomyces cerevisiae. *J Biol Chem*, **271**, 20551-20558.

Rotman, G. and Shiloh, Y. (1999) ATM: a mediator of multiple responses to genotoxic stress. *Oncogene*, **18**, 6135-6144.

Sagher, D. and Strauss, B. (1983) Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during in vitro synthesis: uniqueness of adenine nucleotides. *Biochemistry*, **22**, 4518-4526.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*, **74**, 5463-5467.

Schofield, M.J., Lilley, D.M.J. and White, M.F. (1998) Dissection of the sequence specificity of the holliday junction endonuclease CCE1. *Biochemistry*, **37**, 13042.

Schwacha, A. and Kleckner, N. (1997) Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway. *Cell*, **90**, 1123-1135.

Seeberg, E., Eide, L. and Bjoras, M. (1995) The base excision repair pathway. *Trends Biochem. Sci.*, **20**, 391-397.

Seigneur, M., Bidnenko, V., Ehrlich, S.D. and Michel, B. (1998) RuvAB acts at arrested replication forks. *Cell*, **95**, 419-430.

Sharples, G.J. (2001) The X philes: structure-specific endonucleases that resolve Holliday junctions. *Mol Microbiol*, **39**, 823-834.

Sharples, G.J., Chan, S.N., Mahdi, A.A., Whitby, M.C. and Lloyd, R.G. (1994) Processing of intermediates in recombination and DNA repair: identification of a new endonuclease that specifically cleaves Holliday junctions. *Embo J*, **13**, 6133-6142.

Sharples, G.J. and Leach, D.R. (1995) Structural and functional similarities between the SbcCD proteins of Escherichia coli and the RAD50 and MRE11 (RAD32) recombination and repair proteins of yeast. *Mol Microbiol*, **17**, 1215-1217.

Shen, Z., Cloud, K.G., Chen, D.J. and Park, M.S. (1996) Specific interactions between the human RAD51 and RAD52 proteins. *J Biol Chem*, **271**, 148-152.

Shinohara, A., Ogawa, H. and Ogawa, T. (1992) Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. *Cell*, **69**, 457-470.

Shinohara, A. and Ogawa, T. (1998) Stimulation by Rad52 of yeast Rad51-mediated recombination. *Nature*, **391**, 404-407.

Shinohara, A., Shinohara, M., Ohta, T., Matsuda, S. and Ogawa, T. (1998) Rad52 forms ring structures and co-operates with RPA in single-strand DNA annealing. *Genes Cells*, **3**, 145-156.

Smerdon, M.J. and Thoma, F. (1990) Site-specific DNA repair at the nucleosome level in a yeast minichromosome. *Cell*, **61**, 675-684.

Solinger, J.A., Lutz, G., Sugiyama, T., Kowalczykowski, S.C. and Heyer, W.D. (2001) Rad54 protein stimulates heteroduplex DNA formation in the synaptic phase of DNA strand exchange via specific interactions with the presynaptic Rad51 nucleoprotein filament. *J Mol Biol*, **307**, 1207-1221.

Stasiak, A.Z., Larquet, E., Stasiak, A., Muller, S., Engel, A., Van Dyck, E., West, S.C. and Egelman, E.H. (2000) The human Rad52 protein exists as a heptameric ring. *Curr Biol*, **10**, 337-340.

Sugawara, N. and Haber, J.E. (1992) Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. *Mol Cell Biol*, **12**, 563-575.

Sugiyama, T., New, J.H. and Kowalczykowski, S.C. (1998) DNA annealing by RAD52 protein is stimulated by specific interaction with the complex of replication protein A and single-stranded DNA. *Proc Natl Acad Sci U S A*, **95**, 6049-6054.

Sun, H., Treco, D. and Szostak, J.W. (1991) Extensive 3'-overhanging, single-stranded DNA associated with the meiosis-specific double-strand breaks at the ARG4 recombination initiation site. *Cell*, **64**, 1155-1161.

Sung, P. (1994) Catalysis of ATP-dependent homologous DNA pairing and strand exchange by yeast RAD51 protein. *Science*, **265**, 1241-1243.

Sung, P. (1997a) Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. *J Biol Chem*, **272**, 28194-28197.

Sung, P. (1997b) Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev*, **11**, 1111-1121.

Sung, P. and Robberson, D.L. (1995) DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell*, **82**, 453-461.

Swagemakers, S.M., Essers, J., de Wit, J., Hoeijmakers, J.H. and Kanaar, R. (1998) The human RAD54 recombinational DNA repair protein is a double-stranded DNA-dependent ATPase. *J Biol Chem*, **273**, 28292-28297.

Sweder, K.S. and Hanawalt, P.C. (1992) Preferential repair of cyclobutane pyrimidine dimers in the transcribed strand of a gene in yeast chromosomes and plasmids is dependent on transcription. *Proc Natl Acad Sci U S A*, **89**, 10696-10700.

Symington, L.S. (1998) Homologous recombination is required for the viability of rad27 mutants. *Nucleic Acids Res*, **26**, 5589-5595.

Symington, L.S. (2002) Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol Mol Biol Rev*, **66**, 630-670.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J. and Stahl, F.W. (1983) The double-strand-break repair model for recombination. *Cell*, **33**, 25-35.

Tan, T.L., Essers, J., Citterio, E., Swagemakers, S.M., de Wit, J., Benson, F.E., Hoeijmakers, J.H. and Kanaar, R. (1999) Mouse Rad54 affects DNA conformation and DNA-damage-induced Rad51 foci formation. *Curr Biol*, **9**, 325-328.

Thaler, D.S. and Stahl, F.W. (1988) DNA double-chain breaks in recombination of phage lambda and of yeast. *Annu Rev Genet*, **22**, 169-197.

Tishkoff, D.X., Filosi, N., Gaida, G.M. and Kolodner, R.D. (1997) A novel mutation avoidance mechanism dependent on S. cerevisiae RAD27 is distinct from DNA mismatch repair. *Cell*, **88**, 253-263.

Trujillo, K.M. and Sung, P. (2001) DNA structure-specific nuclease activities in the Saccharomyces cerevisiae Rad50*Mre11 complex. *J Biol Chem*, **276**, 35458-35464.

Tsubouchi, H. and Ogawa, H. (1998) A novel mre11 mutation impairs processing of double-strand breaks of DNA during both mitosis and meiosis. *Mol Cell Biol*, **18**, 260-268.

Tsukamoto, Y., Kato, J. and Ikeda, H. (1996) Hdf1, a yeast Ku-protein homologue, is involved in illegitimate recombination, but not in homologous recombination. *Nucleic Acids Res*, **24**, 2067-2072.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M., Johnston, M., Fields,

S. and Rothberg, J.M. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature*, **403**, 623-627.

Usui, T., Ohta, T., Oshiumi, H., Tomizawa, J., Ogawa, H. and Ogawa, T. (1998) Complex formation and functional versatility of Mre11 of budding yeast in recombination. *Cell*, **95**, 705-716.

Van Komen, S., Petukhova, G., Sigurdsson, S., Stratton, S. and Sung, P. (2000) Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. *Mol Cell*, **6**, 563-572.

van Steeg, H. and Kraemer, K.H. (1999) Xeroderma pigmentosum and the role of UV-induced DNA damage in skin cancer. *Mol Med Today*, **5**, 86-94.

Voelkel-Meiman, K. and Roeder, G.S. (1990) Gene conversion tracts stimulated by HOT1-promoted transcription are long and continuous. *Genetics*, **126**, 851-867.

Wang, Z. and Rossman, T. G. (1994) Isolation of DNA fragments from agarose gel by centrifugation. *Nucleic Acids Res,* **22**, 2862-2863.

Ward, J.F. (1988) DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. *Prog Nucleic Acid Res Mol Biol*, **35**, 95-125.

Whitby, M.C. and Dixon, J. (1997) A new Holliday junction resolving enzyme from Schizosaccharomyces pombe that is homologous to CCE1 from Saccharomyces cerevisiae. *J Mol Biol*, **272**, 509-522.

Whitby, M.C., Osman, F. and Dixon, J. (2002) Cleavage of model replication forks by fission yeast Mus81-Eme1 and budding yeast Mus81-Mms4. *J Biol Chem*.

White, M.F., Giraud-Panis, M.J., Pohler, J.R. and Lilley, D.M. (1997) Recognition and manipulation of branched DNA structure by junction-resolving enzymes. *J Mol Biol*, **269**, 647-664.

White, M.F. and Lilley, D.M. (1997) The resolving enzyme CCE1 of yeast opens the structure of the four-way DNA junction. *J Mol Biol*, **266**, 122-134.

Wilson, D.M. and Thompson, L.H. (1997) Life without DNA repair. *Proc Natl Acad Sci U S A*, **94**, 12754-12757.

Wilson, T.E., Grawunder, U. and Lieber, M.R. (1997) Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature*, **388**, 495-498.

Wu, T.C. and Lichten, M. (1994) Meiosis-induced double-strand break sites determined by yeast chromatin structure. *Science*, **263**, 515-518.

Wu, X. and Wang, Z. (1999) Relationships between yeast Rad27 and Apn1 in response to apurinic/apyrimidinic (AP) sites in DNA. *Nucleic Acids Res*, **27**, 956-962.

Xiao, W., Chow, B.L., Broomfield, S. and Hanna, M. (2000) The Saccharomyces cerevisiae RAD6 group is composed of an error-prone and two error-free postreplication repair pathways. *Genetics*, **155**, 1633-1641.

Xiao, W., Chow, B.L. and Milo, C.N. (1998) Mms4, a putative transcriptional (co)activator, protects Saccharomyces cerevisiae cells from endogenous and environmental DNA damage. *Mol Gen Genet*, **257**, 614-623.

Xiao, W. and Samson, L. (1993) In vivo evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells. *Proc Natl Acad Sci U S A*, **90**, 2117-2121.

Zhu, J., Petersen, S., Tessarollo, L. and Nussenzweig, A. (2001) Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Curr Biol*, **11**, 105-109.